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THE PROTECTIVE PROPERTIES OF THE ALPHA ANTITOXIN AND THETA ANTIHAEMOLYSIN OCCURRING IN *CL. WELCHII* TYPE A ANTISERUM.*

D. G. EVANS.

From the National Institute for Medical Research, London, N.W. 3.

Received for publication April 5, 1943.

THERE are at least two antigenic substances in the filtrates from the growth of *Cl. welchii* type A in nutrient broth. One is a typical toxin, lethal for mice when administered in small doses by intravenous injection, dermonecrotic for guinea-pigs, and haemolytic *in vitro* for sheep's red cells (Glenny, Barr, Llewellyn-Jones, Dalling and Ross, 1933). It reacts with human serum and with egg yolk solution to give a characteristic turbidity (Seiffert, 1939; Nagler, 1939; Macfarlane, Oakley and Anderson, 1941; van Heyningen, 1941). The haemolytic activity of this toxin is not affected by oxygen and, like the reaction with human serum and egg yolk, calcium ions are required for its full development (Macfarlane, Oakley and Anderson, 1941; Oakley and Warrack, 1941). These various reactions are due to one substance, α toxin, and are neutralized by α antitoxin. The second antigenic substance is a haemolysin, known as θ haemolysin, which has properties distinct from those of α toxin. It shows a slight but irregular toxic action when administered to mice, and produces a haemorrhagic reaction, but no necrosis, when injected intradermally into guinea-pigs. It gives no reaction with human serum or egg yolk solution. Its main characteristic is its ability to haemolyse sheep's red cells, but this substance, in contrast with α toxin, is reversibly oxidizable and its action is independent of the presence of calcium ions. θ haemolysin is neutralized by its specific antihaemolysin and also by high titre streptolysin O antiserum and, conversely, θ antihaemolysin neutralizes streptolysin O (Todd, 1941). θ antihaemolysin occurs in the serum of mice, guinea-pigs, ferrets, dogs, cats, rabbits, horses and men (Oakley and Warrack, 1941).

It has long been recognized that α toxin plays an important part in the pathogenic action of *Cl. welchii* type A, but little is known of the significance of θ haemolysin in infection, or of the value or importance of θ antihaemolysin in its control. The antisera prepared for the prophylaxis and therapy of gas gangrene due to infection by *Cl. welchii* type A contain both α antitoxin and θ antihaemolysin, but only their content of the former antibody is determined, in relation to the international standard for α antitoxin, and expressed in international units. If θ haemolysin plays a significant role in the production of gas gangrene due to *Cl. welchii* type A, its antibody may

* The nomenclature used in this paper for the antigens produced by *Cl. welchii* type A is that adopted by the conference held at Cambridge, April 5, 1941 (see *Nature*, 1941, **149**, 56).

also be important in the control of the disease, and it would then be desirable to ensure that the content of θ antihaemolysin in antisera was adequate ; a standard preparation would have to be established and methods of assay devised.

The experiments described below, in which the effects of α antitoxin and θ antihaemolysin, separately and combined, on the development of gas gangrene experimentally produced in the guinea-pig by four different strains of *Cl. welchii* type A have been investigated, were carried out with the object of determining whether θ antihaemolysin played an essential part in the control of the infectious process.

METHODS.

Guinea-pigs weighing from 400 to 500 g. were used throughout. Experimental gas gangrene infection by *Cl. welchii* type A was produced by a method similar to that described by Armstrong and Rae (1941). Into the shaven thigh of the left hind leg of the animal, 0·2 c.c. of a 15 per cent. solution of $CaCl_2$ in distilled water was injected intramuscularly. Three hours later, 0·2 c.c. of a suspension of washed *Cl. welchii* organisms was injected into the same site. Four strains have been used, S.R. 9, S. 107, B.S.I. and "Corcoran," and all have produced typical gangrenous lesions, followed, in the case of the more virulent strains, by death of the guinea-pig. Animals which received $CaCl_2$ solution and no subsequent injection of bacilli showed, 24 hours later, a slight swelling at the site of inoculation, but at 48 hours no reaction at all was perceptible. In each experiment the suspension of washed bacilli was prepared in the same way. The organisms were grown in liver broth for three hours ; the culture was then centrifuged, the supernatant fluid removed and the deposit, after being washed once in saline, was resuspended in saline to give a concentration, by measured opacity, of approximately 250 million organisms per c.c. From this saline suspension ten-fold dilutions were made and 0·2 c.c. quantities of the different dilutions were injected immediately.* Strains S.R. 9 and B.S.I. proved to be the most virulent ; on the numerous occasions on which they have been tested it has been found that 0·2 c.c. of the original saline suspensions, diluted 10^{-6} and 10^{-7} respectively, have produced fatal infection. These doses calculated from the concentration of the initial suspensions were equivalent to 50 and 5 organisms respectively, and that the doses were of this order was confirmed by plate counts. Strain S. 107 was not so virulent as S.R. 9 and B.S.I., since it was necessary to inject 0·2 c.c. of a 10^{-3} dilution of the original suspension to produce fatal infection. This dose was equivalent to 50,000 organisms, but much smaller doses (50 organisms) produced severe infection with survival or greatly delayed death. "Corcoran" strain was even less virulent, since 0·2 c.c. of the undiluted original suspension (50 million organisms) did not kill guinea-pigs regularly, although the animals became infected and exhibited well-marked and typical lesions. Even when larger doses than this were employed, death occurred only occasionally. Stephenson and Ross (1940), using mice, reported that this strain was more effective when given intraperitoneally rather than intramuscularly. Using guinea-pigs, however, this did not prove to be the case ; infection by the intraperitoneal route was somewhat less effective than by the intramuscular route and, accordingly, the latter method of infecting the animals was adopted.

Three *Cl. welchii* type A antisera, all originally prepared at the Wellcome Physiological Research Laboratories at Beckenham, have been investigated for their activity

* No attempt has been made to determine the average lethal dose for each of the strains, but in all the comparative tests unprotected animals were injected with varying doses of organisms and, in order to indicate the relative protective properties of the antisera investigated, the smallest dose causing death in the control series is referred to, for convenience, as the lethal dose.

in protecting guinea-pigs against the gas gangrene produced by infection with *Cl. welchii* type A. The particulars of these three sera are :

(1) LX241 : A refined and purified antiserum prepared in a horse, and containing 100 units of α antitoxin, and only 0·3 units of θ antihaemolysin,* per c.c.

(2) R5432 : A natural unconcentrated antiserum prepared in a horse, and containing 120 units of θ antihaemolysin, and only 0·2 units of α antitoxin, per c.c.

(3) The International Standard Gas Gangrene Antitoxin (perfringens) : A natural unconcentrated serum prepared in a horse and converted into the dry stable condition at the National Institute for Medical Research, Hampstead, in 1930 and adopted as the International Standard in 1931. For the purposes of these experiments the dry standard was dissolved in saline and the volume adjusted so that 1 c.c. contained 100 units of α antitoxin (and 100 units of θ antihaemolysin).

The protective action of the two sera, LX241 and R5432, given together as a mixture has also been investigated.

All the experiments have been carried out on the same general plan. In the case of each serum injection, the serum was diluted in saline, so that the required dose was contained in a volume of 2 c.c., and this was injected subcutaneously above the site where the injection of washed organisms was to be made. The serum was injected three hours before the infecting dose of organisms was given.

RESULTS.

Comparison of the Protective Action of θ Antihaemolysin and α Antitoxin.

In each comparative test three groups of guinea-pigs, 6 to 8 animals in each group, were used. To the guinea-pigs in one group no serum was given, to each of those in another 150 units of θ antihaemolysin, and those in the third group each received 50 units of α antitoxin. At the same time 0·2 c.c. of the calcium chloride solution was given to each animal. Three hours later the doses of washed organisms shown in Table I were injected and the animals were observed for 7 days. A typical protocol is presented in Table I, from which it will be seen that 50 units of α antitoxin protected the guinea-pigs against very many lethal, or infecting, doses of *Cl. welchii* type A. No end-point was reached with strain S.R. 9, but the guinea-pig which received 1000 lethal doses survived and showed only a slight local reaction at the site of injection. With strain B.S.I. protection was afforded against 10,000, but not against 100,000, lethal doses ; while in the case of strain S. 107, 50 units of α antitoxin protected against 100 lethal doses, which was the largest dose of this strain which was administered. The protective action of α antitoxin against the infection produced by the strain " Corcoran " was also demonstrated, since no reaction occurred in any of the guinea-pigs protected with 50 units of α antitoxin, although they received 100,000 times the smallest dose of organisms which produced a local reaction in the unprotected controls.

On the other hand, 150 units of θ antihaemolysin gave no protection whatever against the smallest lethal or infecting dose of organisms administered, little or no difference being observed, with each of the four strains, between the groups of guinea-pigs receiving the θ antihaemolysin and the corresponding groups of unprotected controls.

* No standard for θ antihaemolysin has yet been established, and no unit of activity has been defined. Accordingly, as a standard of reference for the purposes of these experiments, we have assigned to the standard gas gangrene antitoxin (perfringens) solution which is issued in the form of a glycerol-saline solution by the National Institute, Hampstead, and which contains 20 units of α antitoxin per c.c., the value of 20 units of θ antihaemolysin per c.c. This provisional unit has been used for some time at the Wellcome Physiological Research Laboratories in investigations with θ haemolysin.

The effectiveness of α antitoxin in protecting guinea-pigs against the sequelae following the infection with *Cl. welchii* type A, and the complete failure of θ antihaemolysin to afford any such protection, is brought out in an even more striking way when the nature and composition of the two antisera are compared. The antiserum LX241 was a highly purified preparation. It contained only 0·73 mg. of nitrogen, and 12·5 mg. of total solids, per c.c. Assuming that all the nitrogen of the preparation is present as protein, 1 c.c. of this preparation contains about 4·7 mg. of protein. The protection provided by 50 units of α antitoxin against very many lethal or infecting doses of the four strains of *Cl. welchii* type A is therefore associated with 0·365 mg. of nitrogen, 6·25 mg. of total solids or 2·35 mg. of protein. The antiserum R5432 was a natural unconcentrated preparation. It contained 13·1 mg. of nitrogen and 96·0 mg. of total solids per c.c.; assuming that all the nitrogen is present as protein, 1 c.c. of this preparation contains about 84 mg. of protein. The volume of serum R5432 administered to each guinea-pig was 1·25 c.c.; in contrast with the α antitoxin therefore the quantity of θ antihaemolysin associated with nearly 50 times as much nitrogen, protein or solid matter (viz. 16·4 mg. nitrogen, 120 mg. of total solids or 105 mg. of protein) as the α antitoxin, failed to give protection against the smallest lethal or infecting dose of organisms.

The Protective Action of θ Antihaemolysin and α Antitoxin Combined.

The following experiments were carried out to see whether θ antihaemolysin enhanced the protective action of α antitoxin. The two most virulent strains, B.S.I. and S.R. 9, were used in these comparative tests. Two groups of guinea-pigs were employed; the animals in one group were given varying doses of the serum LX241 (α antitoxin) alone, and those of the other group were given the same doses of LX241 to which 1·25 c.c. of serum R5432 (i.e. 150 units of θ antihaemolysin) had been added. Three hours after the injection of serum each guinea-pig was injected with the same dose of washed organisms which, as shown by tests on unprotected animals carried out at the same time with the same parent suspension, was equivalent to at least 10 lethal doses. The results (Table II) show that protection was afforded by 5 units and 10 units of α antitoxin against 10 lethal doses of S.R. 9 and B.S.I. respectively, and no improvement in the protection was obtained when the α antitoxin was given together with 150 units of θ antihaemolysin. Indeed it appeared, especially in the case of strain S.R. 9, that the addition of the θ antihaemolysin had an inhibitory effect on the protective action of the α antitoxin, since the mixed sera in doses containing more than 5 units of α antitoxin failed to save the lives of guinea-pigs injected with 10 lethal doses of this strain. This unexpected result was investigated further.

The Effect of Normal Horse Serum on the Protective Action of α Antitoxin.

In addition to the results reported above, it has been found on several occasions during this investigation that θ antihaemolysin (R5432) reduced the protective power of α antitoxin (LX241) when the two were mixed together and given as a single injection; in controlled experiments a dose of α antitoxin which afforded complete protection when given alone, failed to protect when given with θ antihaemolysin. It was thought that the results observed might be due to the possibility that serum LX241, being a highly purified preparation and relatively free from extraneous non-specific, substances, might be less readily absorbed when it was injected with a large quantity of foreign protein, such as the horse serum which contained the θ antihaemolysin. Experiments were therefore carried out in which the protective action of α antitoxin (LX241) diluted in saline was compared with its action when similarly diluted in normal

TABLE I.—*The Resistance of Guinea-pigs to Varying Doses of* Cl. welchii *type A* (*Four Different Strains*) *after Receiving* (a) *No Serum*, (b) 150 *Units of* θ *Antihaemolysin and* (c) 50 *Units of* α *Antitoxin.*

Dose of *Cl. welchii* suspension.	Strain S.R. 9.			Strain B.S.I.			Strain S. 107.			Strain "Corcoran."		
	No serum.	150 units θ anti-haemolysin.	50 units α anti-toxin.	No serum.	150 units θ anti-haemolysin.	50 units α anti-toxin.	No serum.	150 units θ anti-haemolysin.	50 units α anti-toxin.	No serum.	150 units θ anti-haemolysin.	50 units α anti-toxin.
0·2 c.c. undiluted	..	..	..	..	..	..	..	..	..	D. 4 dys.	S. (s.g.)	S. (n.r.)
,, 10^{-1} dilution	..	..	..	..	..	..	D. 36 hrs.	D. 3 dys.	S. (l.r.)	S. (s.g.)	S. (s.g.)	S. (n.r.)
,, 10^{-2} ,,	..	..	..	D. 2 dys.	D. 3 dys.	D. 4 dys.	D. 4 dys.	D. 7 dys.	S. (n.r.)	S. (s.g.)	D. 6 dys.	S. (n.r.)
,, 10^{-3} ,,	D. 20 hrs.	D.< 40 hrs.	S. (l.r.)	D. 3 dys.	D. 3 dys.	S. (l.r.)	D. 4 dys.	D. 4 dys.	S. (n.r.)	S. (m.g.)	S. (m.g.)	S. (n.r.)
,, 10^{-4} ,,	D. 3 dys.	D.< 40 hrs.	S. (n.r.)	D. 3 dys.	D. 4 dys.	S. (l.r.)	S. (s.g.)	S. (s.g.)	S. (n.r.)	S. (s.g.)	S. (m.g.)	S. (n.r.)
,, 10^{-5} ,,	D. 20 hrs.	D.< 40 hrs.	S. (n.r.)	D. 3 dys.	D. 3 dys.	S. (n.r.)	S. (s.g.)	S. (s.g.)	S. (n.r.)	S. (l.r.)	S. (l.r.)	S. (n.r.)
,, 10^{-6} ,,	D. 3 dys.	D. 6 dys.	S. (n.r.)	D. 2 dys.	D. 3 dys.	S. (n.r.)	S. (m.g.)	S. (s.g.)	S. (n.r.)	S. (n.r.)	S. (n.r.)	S. (n.r.)
,, 10^{-7} ,,	S. (l.r.)	S. (s.g.)	S. (n.r.)	D. 3 dys.	S. (s.g.)	S. (n.r.)	S. (m.g.)	S. (m.g.)	S. (n.r.)	..	..	..
,, 10^{-8} ,,	S. (n.r.)	S. (n.r.)	S. (n.r.)	S. (n.r.)	S. (n.r.)	S. (n.r.)	S. (n.r.)	S. (n.r.)	S. (n.r.)	..	..	..

Dilutions made from suspensions having a concentration of 250 million organisms per c.c.
Key for Tables I to V: D. = death; S. = survival; n.r. = no reaction; l.r. = local reaction; m.g. = moderate gangrene; s.g. = severe gangrene.

TABLE II.—*The Resistance of Guinea-pigs to* 10 *Lethal Doses of* Cl. welchii *type A* (*Two Strains*) *after Receiving* (a) α *Antitoxin and* (b) *a Mixture of* α *Antitoxin and* θ *Antihaemolysin.*

Strain S.R. 9.			Strain B.S.I.		
Units of α antitoxin.	Result.	Units of α antitoxin and θ antihaemolysin.	Result.	Units of α antitoxin.	Result.	Units of α antitoxin and θ antihaemolysin.	Result.
40 units	S. (n.r.)	40 units α 150 ,, θ	D. 2 dys.	40 units	S. (n.r.)	40 units α 150 ,, θ	S. (l.r.)
20 ,,	S. (n.r.)	20 ,, α 150 ,, θ	S. (s.g.)	20 ,,	S. (n.r.)	20 ,, α 150 ,, θ	S. (l.r.)
10 ,,	S (n.r.)	10 ,, α 150 ,, θ	D. 2 dys.	10 ,,	S. (l.r.)	10 ,, α 150 ,, θ	S. (s.g.)
5 ,,	S. (n.r.)	5 ,, α 150 ,, θ	D. 2 dys.	5 ,,	D. 3 dys.	5 ,, α 150 ,, θ	D. 24 hrs.
2·5 ,,	D. 2 dys.	2·5 ,, α 150 ,, θ	D. 4 dys.	2·5 ,,	D. 3 dys.	2·5 ,, α 150 ,, θ	D. 24 hrs.
1·25 ,,	D. 2 dys.	1·25 ,, α 150 ,, θ	D. 24 hrs.	1·25 ,,	D. 3 dys.	1·25 ,, α 150 ,, θ	D. 2 dys.

horse serum. The normal horse serum used in the test contained 12·8 mg. nitrogen and 98 mg. of total solids per c.c., and was thus very similar in character to the serum R5432. The experiments were carried out in an exactly similar way to those described above, the strains B.S.I. and S.R. 9 again being used. The results (Table III) show that α antitoxin diluted in saline exhibited a superior protective property than when diluted in normal horse serum against 10 lethal doses of *Cl. welchii* type A. With both strains, while 40 units of α antitoxin diluted in horse serum failed to protect guinea-pigs against extensive gangrene, and 10 units similarly administered failed to protect against death, when diluted in saline 5 units of α antitoxin protected guinea-pigs completely against 10 lethal doses of S.R. 9 and 10 units protected guinea-pigs almost completely against 10 lethal doses of B.S.I.

The inhibitory effect of the serum R5432, referred to above, would appear to be due therefore not to the θ antihaemolysin as such, but to the constituents of the horse serum in which it is contained; and these results demonstrate the advantages of refined and purified antitoxins.

TABLE III.—*The Resistance of Guinea-pigs to* 10 *Lethal Doses of* Cl. welchii *type A (Two Strains) after Receiving α Antitoxin* (a) *Diluted in Saline and* (b) *Diluted in Normal Horse Serum.*

Units of α antitoxin.	Strain S.R. 9.		Strain B.S.I.	
	Antitoxin diluted in saline.	Antitoxin diluted in horse-serum.	Antitoxin diluted in saline.	Antitoxin diluted in horse-serum.
40 units	S. (n.r.)	S. (s.g.)	S. (n.r.)	S. (s.g.)
20 „	S. (n.r.)	S. (s.g.)	S. (n.r.)	S. (s.g.)
10 „	S. (n.r.)	D. 3 dys.	S. (l.r.)	D. 3 dys.
5 „	S. (n.r.)	D. 2 dys.	D. 3 dys.	D. 20 hrs.
2·5 units	D. 2 dys.	D. 2 dys.	D. 2 dys.	D. 3 dys.
1·25 „	D. 2 dys.	D. 2 dys.	D. 3 dys.	D. 20 hrs.

The Protective Action of the International Standard Gas Gangrene Antitoxin (perfringens).

Experiments were carried out to determine whether the international standard preparation of gas gangrene antitoxin (perfringens) protected guinea-pigs against infection with strains of *Cl. welchii* type A. Only the two most virulent strains, B.S.I. and S.R. 9, were used in these experiments.

In one series of experiments a control group of guinea-pigs was injected with a wide range of infecting doses, and a similar group was injected at the same time with the same doses of the organism three hours after each animal had received a dose of the international standard containing 50 units of α antitoxin. This quantity of the standard also contained 50 units of θ antihaemolysin but, as the experiments described above have shown, this component of the antiserum plays no part in the protection of the guinea-pig against infection by *Cl. welchii* type A. The results are summarized in Table IV, from which it will be seen that the quantity of the international standard injected protected guinea-pigs against 10,000 lethal doses of each of the strains B.S.I. and S.R. 9. These results suggest that the international standard possesses protective properties similar to those exhibited by the highly refined α antitoxin LX241.

In a second series of experiments the international standard and the highly refined α antitoxin LX241 were directly compared on the basis of their content of α antitoxin. Guinea-pigs in one group were each given a dose of the standard preparation containing 50 units of α antitoxin, and each of those in a corresponding group were given a dose of the antiserum LX241 also containing 50 units of α antitoxin. Three hours later the animals of both groups, as well as those comprising the control group of unprotected animals, were given the doses of the strain of B.S.I. shown in Table V.

TABLE IV.—*The Resistance of Guinea-pigs to Varying Doses of* Cl. welchii *type A (Two Strains) after Receiving* (a) *No Serum and* (b) 50 *Units of the International Standard Gas Gangrene Antitoxin (perfringens).*

Dose of *Cl. welchii* suspension.	Strain S.R. 9.		Strain B.S.I.	
	No serum.	International Standard, 50 units.	No serum.	International Standard, 50 units.
0·2 c.c. 10^{-1} dilution	..	D. 3 dys.	..	D. 3 dys.
,, 10^{-2} ,,	..	S. (l.r.)	..	D. 6 dys.
,, 10^{-3} ,,	D. 4 dys.	S. (l.r.)	D. 24 hrs.	S. (n.r.)
,, 10^{-4} ,,	D. 24 hrs.	S. (n.r.)	D. 2 dys.	S. (n.r.)
,, 10^{-5} ,,	D. 4 dys.	S. (n.r.)	D. 2 dys.	S. (n.r.)
,, 10^{-6} ,,	D. 2 dys.	S. (n.r.)	D. 7 dys.	S. (n.r.)
,, 10^{-7} ,,	S. (l.r.)	S. (n.r.)	D. 2 dys.	S. (n.r.)
,, 10^{-8} ,,	S. (n.r.)	S. (n.r.)	S. (n.r.)	S. (n.r.)

Dilutions made from suspensions having a concentration of 250 million organisms per c.c.

TABLE V.—*The Resistance of Guinea-pigs to Varying Doses of* Cl. welchii *type A (Strain B.S.I.) after Receiving* (a) *No Serum,* (b) 50 *Units of the International Standard Gas Gangrene Antitoxin (perfringens) and* (c) 50 *Units of Serum LX*241.

Dose of *Cl. welchii* suspension.	No serum.	International Standard, 50 units.	Serum LX241, 50 units α antitoxin.
0·2 c.c. 10^{-1} dilution	..	S. (s.g.)	S. (s.g.)
,, 10^{-2} ,,	..	S. (m.g.)	S. (s.g.)
,, 10^{-3} ,,	..	S. (n.r.)	S. (n.r.)
,, 10^{-4} ,,	..	S. (n.r.)	S. (n.r.)
,, 10^{-5} ,,	D. 3 dys.	S. (n.r.)	S. (n.r.)
,, 10^{-6} ,,	D. 2 dys.	S. (n.r.)	S. (n.r.)
,, 10^{-7} ,,	D. 3 dys.	S. (n.r.)	S. (n.r.)
,, 10^{-8} ,,	S. (n.r.)	S. (n.r.)	S. (n.r.)

Dilutions made from a suspension having a concentration of 250 million organisms per c.c.

It will be seen that the two sera exhibited an almost identical behaviour in regard to their power of protecting guinea-pigs against infection ; the amount of the serum LX241 injected contained, as judged by independent assay in relation to the standard, 50 units of α antitoxin, and protected guinea-pigs in exactly the same way as 50 units of α antitoxin contained in the international standard preparation.

DISCUSSION.

The preparations of gas gangrene antitoxin provided for the prophylaxis and treatment of infections due to *Cl. welchii* type A are assayed for their content of α antitoxin. The assay is performed by means of toxins and it is precise. The question has been raised as to whether the real value of gas gangrene antitoxin, for prophylaxis or therapy, is entirely represented by its antitoxin content. While that question is not answered by the results of these experiments, since the possible value of a purely anti-bacterial serum has not been investigated, it is considered that evidence has been produced to show, firstly that α antitoxin plays some part in preventing infection in experimental gas gangrene due to *Cl. welchii* type A, and secondly, that θ antihaemolysin does not. Moreover, the results of the experiments summarized in Tables IV and V suggest that further information concerning the properties of the gas gangrene antitoxins provided for field use might be obtained by comparative tests, not only against toxin, but against living cultures as well. A precise assay against living organisms is not to be expected, but comparative tests on lines similar to those indicated might show to what extent antitoxin content and the power to protect against living organisms

are correlated. The close similarity of behaviour of the two preparations of α antitoxin used in these experiments suggests that this correlation may be closer than has hitherto been supposed.

SUMMARY.

1. Gas gangrene has been regularly produced in guinea-pigs by the injection of washed cultures from four strains of *Cl. welchii* type A. The four strains used varied in their relative virulence, two being of high, one of intermediate, and one of low virulence.

2. A *Cl. welchii* type A antiserum with a high content of α antitoxin and containing only a trace of θ antihaemolysin was highly effective in protecting guinea-pigs against infection.

3. A *Cl. welchii* type A antiserum with a high content of θ antihaemolysin and containing only a trace of α antitoxin was quite ineffective in protecting guinea-pigs against infection.

4. The *Cl. welchii* type A antiserum which serves as the International Standard Gas Gangrene Antitoxin (perfringens) protected guinea-pigs against experimental gas gangrene produced by the injection of *Cl. welchii* type A as effectively as the antiserum which contained mainly α antitoxin and only a trace of θ antihaemolysin.

5. The protective action of α antitoxin was not enhanced, but on the contrary was reduced, when it was injected with the θ antihaemolysin preparation used in these experiments. Further investigation showed that a similar inhibitory effect was produced when the highly purified preparation of α antitoxin was injected with normal horse serum. It is suggested that these results may be due to a reduction in the rate of absorption of α antitoxin when it is injected with the large quantities of non-specific material present in horse serum.

The author is greatly indebted to Dr. R. F. Montgomerie and Miss H. E. Ross, of the Wellcome Physiological Research Laboratories, for their valuable help and criticism and for providing the sera which have been used in this investigation.

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AN EXAMINATION OF THE RELATIONSHIP BETWEEN THE BACTERIOSTATIC ACTIVITY AND THE NORMAL REDUCTION POTENTIALS OF SUBSTITUTED QUINONES.

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SEVERAL workers have suggested that there might be a relationship between the oxidation-reduction potential of a substance and its chemotherapeutic activity. Breyer (1939), for example, considered that because quinquevalent arsenic compounds act only after being reduced to the trivalent form, the reduction potentials of such quinquevalent compounds might be related to their biological activities. He therefore measured the half-wave potentials of a series of phenylarsonic acids and found that, although they were all reduced with difficulty, the introduction of substituents decreased the ease of reduction. No correlation between reduction potential and toxicity was, however, observed.

Dubos (1929) attempted to obtain evidence in support of the hypothesis that "methylene blue and oxidized indophenols are bacteriostatic because they 'poise' the medium at an oxidation potential outside the range in which pneumococcus and human and bovine strains of haemolytic streptococci can grow." Although the evidence he obtained is not inconsistent with this hypothesis, his results can be explained equally well in other ways, as he himself admits.

A somewhat similar concept, although expressed in different terms, was put forward by Shinn, Main and Mellon (1938) and others, who suggested that the antibacterial action of the sulphonamides was due to their conversion to an anticatalase. This, by inhibiting the destruction of hydrogen peroxide, leads to the building up of a concentration that the streptococcus and pneumococcus cannot tolerate.

An elaboration of Dubos' hypothesis was advanced by Fildes (1940). Anaerobes will not grow if the oxidation-reduction potential of the medium is maintained at a certain level (Fildes, 1929; Knight and Fildes, 1930), such conditions presumably being incompatible with the anaerobic metabolism of such organisms, and Fildes suggested that the inhibiting effect of thionine and methylene blue on aerobes might be the result of a similar action. He postulated that the anaerobic conditions within the cell are upset by the diffusion into it of the oxidizing dye, resulting in aerobic conditions. This implies that only diffusible dyes will be antibacterial, a non-diffusible dye with a similar oxidizing capacity being inactive. Fildes suggested that the antibacterial action of such diffusible dyes is due to "interference by oxidation with an essential metabolite required to be reduced in order that an enzyme associated with it might function normally as in growth."

Finally, in order to explain chemotherapeutic interference, von Jancsó and von Jancsó (1936) advanced the theory that the reduction potentials of such oxidation-reduction systems as interfere with the action of chemotherapeutic agents fall within

a definite range. They found (*cf.* Table II) that in a series of oxidation-reduction indicators the substances that interfere with the trypanocidal activity of stibophen and 3-amino-4-hydroxy-arsenoxide have potentials ranging from + 0·115 to — 0·060 volt (pH 7·0), and that the degree of interference varies with the value of the normal potential, a distinct optimum being attained at + 0·011 volt.

The series of highly antibacterial quinones tested under standard conditions by Oxford (1942*b*) provided an opportunity of determining whether oxidation-reduction potential could be correlated with activity against strains of *Staph. aureus* and of *Bact. coli*. Oxford's results indicated that the activity of the parent toluquinone against *Staph. aureus* is markedly enhanced by the introduction of a methoxyl group into the 4-position, and still further increased by the introduction of a second methoxyl group into the 6-position. The introduction of hydroxyl groups, on the other hand, generally reduced the activity, both in toluquinone and in the more active 4-methoxy-toluquinone and 4 : 6-dimethoxytoluquinone. The activity against *Bact. coli* was practically the same in all the compounds, the introduction of a hydroxyl or methoxyl group having little or no effect.

The behaviour at the dropping mercury electrode of a number of mould metabolites and related quinones in acetate buffered 75 per cent. alcoholic solution has been examined by Page and Robinson (1943), and the half-wave potentials versus the saturated calomel electrode (S.C.E.) at 25° C. of the sesubstances have been recorded. We expected that the reduction potentials of these quinones might decrease with increasing bacteriostatic activity, but this was not so, as will be seen from Table I.

The values of E'_o, that is, the oxidation-reduction potentials at pH 7·0 referred to the normal hydrogen electrode, were calculated from the values of the normal reduction potential (E_o), which in turn were calculated from the half-wave potentials following the method of Smith, Kolthoff, Wawzonek and Ruoff (1941). It can be shown theoretically (and has been confirmed experimentally) that in the reversible oxidation of a hydroquinone to a quinone or the reduction of a quinone to a hydroquinone, the half-wave potential shifts about 0·0591 volt per unit decrease of pH. Assuming this value, the potential for a pH of zero was calculated, and, from this, the normal reduction potential was deduced, taking 0·246 v. as the standard potential of the saturated calomel electrode. The value obtained for 2-methyl-1 : 4-naphthaquinone (about + 0·43 v.) is in harmony with the figure of + 0·408 v. obtained by Fieser and Fieser (1935) and of + 0·422 v. obtained by Lugg, Macbeth and Winzer (1936) using the potentiometric method, and with the value of 0·458 v. obtained by McCawley and Gurchot (1940) using a polarographic procedure.

The half-wave potentials of the vitamin K analogues, phthiocol (2-methyl-3-hydroxy-1 : 4-naphthaquinone), phthiocol methyl ether (2-methyl-3-methoxy-1 : 4-naphthaquinone) and 2 : 3-dimethyl-1 : 4-naphthaquinone were measured by the procedure described by Page and Robinson (1943). McCawley and Gurchot (1940) obtained for phthiocol a value of 0·256 v. for E_o, with which our value of 0·26 v. is in excellent agreement. Ball (1934) and Lugg, Macbeth and Winzer (1936) both report a figure of 0·299 v.

The specimens of phthiocol, m.p. 171–173° (Fieser, 1940), phthiocol methyl ether, m.p. 91–93° (Madinaveitia, 1934) and 2 : 3-dimethyl-1 : 4-naphthaquinone, m.p. 122–124° (Fieser and Chang, 1942) were prepared by Dr. J. W. Barnett.

The values of the normal reduction potentials of eight of the quinones have been taken from data determined in the classical way by Conant and Fieser (1924). The potentials are to a limited extent independent of the nature of the solvent, Conant and Fieser (1923) obtaining the same values whether 50 per cent. or 95 per cent. alcohol was used.

TABLE I.—*Comparison of Reduction Potentials of Quinones with Bacteriostatic Activity.*

Compound.	Bacteriostatic activity. Dilutions giving complete inhibition. *Staph. aureus.* No. 3095.*	*Staph. aureus.* No. 3750.*	*Staph. aureus.* No. 3761.*	*Staph. aureus.* No. 663.†	*B. coli.* No. 86.*	*B. coli.* No. 613-†	Half-wave potential (S.C.E.) at pH 6·24.	Normal reduction potential Eo (calc.).	Reduction potential E'o (calc.).
Benzoquinone	..	..	..	< 5,000	..	..	..	+ 0·70‡	+ 0·29
Toluquinone	10,000	10,000	10,000	..	< 10,000	..	+ 0·05	+ 0·65	+ 0·24
6-Bromo-2 : 3 : 5-trimethyl-quinone	..	..	..	10,000	..	..	− 0·03	+ 0·57	+ 0·16
4-methoxy-2 : 5-toluquinone	300,000	400,000	< 100,000	..	10,000	..	− 0·06	+ 0·54	+ 0·13
Pseudocumoquinone	..	..	..	50,000	..	5,000	− 0·07	+ 0·53	+ 0·12
1 : 4-Naphthaquinone	..	..	..	10,000	..	..	..	+ 0·48‡	+ 0·07
2 : 5-Dimethoxybenzoquinone	..	100,000	..	..	< 12,000	..	..	+ 0·47‡	+ 0·06
Phenanthraquinone	..	..	..	500,000	..	..	..	+ 0·47‡	+ 0·06
Duroquinone	..	..	..	10,000	..	< 5,000	− 0·14	+ 0·46	+ 0·05
4 : 6-Dimethoxy-2 : 5-toluquinone	600,000	600,000	600,000	500,000	< 10,000	< 5,000	− 0·16	+ 0·44	+ 0·03
3-Hydroxy-4-methoxy-2 : 5-toluquinone (fumigatin)	50,000	33,000	33,000	..	12,000	..	− 0·16	+ 0·44	+ 0·03
2 : 5-Dihydroxybenzoquinone	< 10,000	< 10,000	< 10,000	..	< 10,000	..	..	+ 0·44‡	+ 0·03
2-Methyl-1 : 4-naphthaquinone	..	..	..	300,000	..	< 5,000	− 0·17	+ 0·43	+ 0·02
2 : 3-Dimethyl-1 : 4-naphthaquinone	..	..	..	100,000	..	..	− 0·25	+ 0·35	− 0·07
3 : 6-Dihydroxy-4-methoxy-2 : 5-toluquinone (spinulosin)	6,000	6,000–17,000	6,000	..	10,000	..	− 0·30	+ 0·30	− 0·11
2-Methyl-3-methoxy-1 : 4-naphthaquinone	..	..	..	5,000	..	< 5,000	− 0·32	+ 0·28	− 0·13
2-Methyl-3-hydroxy-1 : 4-naphthaquinone (phthiocol)	..	..	..	10,000	..	< 5,000	− 0·34	+ 0·26	− 0·15
Disodium anthraquinone-1 : 5-disulphonate	..	..	..	< 5,000	..	..	..	+ 0·24‡	− 0·17
Sodium anthraquinone-1-sulphonate	..	..	..	< 5,000	..	..	..	+ 0·20‡	− 0·22
2-Methyl-anthraquinone	..	..	..	< 5,000	..	..	..	+ 0·15‡	− 0·26

* Determined by Oxford (1942*b*).
† Determined by Dr. J. Ungar.
‡ Determined by Conant and Fieser (1924).

TABLE II.—*Comparison of Reduction Potentials (E'_0 at pH 7·0) of Oxidation-reduction Indicators with Bacteriostatic Activity and with Interference Activity.*

Indicator.	Oxidation-reduction potential (E'_0 at pH 7·0).*	Bacteriostatic activity against *Staph. aureus*. Dilutions giving complete inhibition.	Oxidation-reduction potential.† (E'_0 at pH 7·0.)	Interference with 3-amino-4-hydroxy-phenylarsen-oxide.†	Interference with stibophen.†
Phenol - indo - 2 : 6 - dibromo-phenol	+ 0·218	< 5,000	+ 0·217	0	0
o-Cresol-indophenol	+ 0·191	< 5,000	+ 0·181	0	0
1 - Naphthol - 2 - sodium sulphonate-indo-2 : 6-dichlorophenol	+ 0·118	10,000	+ 0·119	0	±
Tolylene blue	+ 0·115	5,000	+ 0·115	++	++
Lauth's violet (thionine)	+ 0·063	10,000	+ 0·062	++	++
Brilliant cresyl blue	+ 0·047	100,000	+ 0·047	+++	+++
Gallocyanine	+ 0·021	< 5,000	+ 0·021	++	+++
Methylene blue	+ 0·011	50,000	+ 0·011	++	++
Toluidine blue	+ 0·011	100,000	+ 0·011	++++	++++
Azur I	+ 0·011	50,000	+ 0·011	++++	++++
"Janus green"‡	..	..	− 0·035	++	++
Potassium indigo tetrasulphonate	− 0·046	< 5,000	− 0·046	0	0
Potassium indigo trisulphonate	− 0·081	< 5,000	− 0·081	0	0
Nile blue	− 0·122	100,000	− 0·122	0	0
Potassium indigo disulphonate	− 0·125	< 5,000	− 0·125	0	0
Cresyl fast violet	− 0·167	100,000	− 0·167	0	0
Pheno-safranine	− 0·252	50,000	− 0·252	0	0
Janus green‡	− 0·255	500,000	..	..	..
Neutral red	− 0·325	< 5,000	− 0·330	0	0

* Data supplied by manufacturer (B.D.H.).
† Determined by von Jancsó and von Jancsó (1936).
‡ Janus green (B.D.H.) is probably different from that used by von Jancsó and von Jancsó. The potential quoted by the latter is for a colour change of blue to red.

The bacteriostatic data for the four strains of *Staph. aureus* and two strains of *Bact. coli* determined by Oxford (1942*b*) and by Dr. J. Ungar refer to the glucose-broth cultures after 20 hours' incubation.

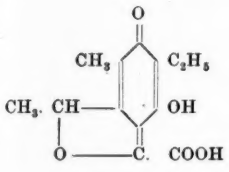
It is worth noting that the relatively high antibacterial activity of 2-methyl-1 : 4-naphthaquinone is reduced considerably by the introduction of a hydroxyl-group in the 3-position. It is reduced still further if the substituent is a methoxyl group, in striking contrast to Oxford's findings in the toluquinone series.

It will be seen from the results that the introduction of a methoxyl or hydroxyl group into toluquinone decreases the reduction potential, the effect of two methoxyl groups being approximately equal to that of one methoxyl and one hydroxyl group. This decrease is not, however, accompanied by a corresponding increase in bacteriostatic activity. Again, the reduction potential of 4 : 6-dimethoxytoluquinone is much less than that of 4-methoxytoluquinone, although the bacteriostatic potency of 4 : 6-dimethoxytoluquinone is only slightly greater. On the other hand, 4 : 6-dimethoxytoluquinone and 3-hydroxy-4-methoxytoluquinone (fumigatin) have approximately the same reduction potentials, but widely differing antibacterial powers.

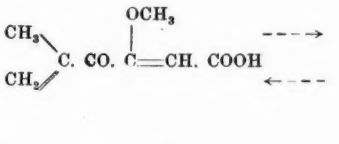
Although no simple relationship appears to exist between the oxidation-reduction potentials of these quinones and their bacteriostatic activities, it was noticed on plotting these values that all the markedly active quinones have potentials falling between − 0·10 and + 0·15 volt at pH 7·0, though not all the quinones with reduction potentials falling within these limits are markedly active. Thus in the naphthaquinone series, the unsubstituted compound is relatively inactive, but on substituting a methyl group in the 2-position, the reduction potential falls and the bacteriostatic activity increases thirty-fold. The substitution of a second methyl group in the 3-position causes the reduction potential to fall further and leads to a

decrease in activity. If this second group is replaced by a methoxyl or hydroxyl group, the reduction potential falls still further and passes outside the potential range for bacteriostatic activity. Optimal activity is associated with a reduction potential of + 0·03 volt. In other words, relatively strong oxidizing or reducing quinones at pH 7·0 are inactive, or nearly so, against *Staph. aureus* in a glucose-broth medium.

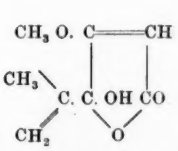
In view of the fact that this range is similar to that observed by von Jancsó and von Jancsó (*vide supra*) for the series of oxidation-reduction indicators which interfere with chemotherapeutic activity, the bacteriostatic activity of eighteen commercial oxidation-reduction indicators (B.D.H.) was tested against *Staph. aureus*. In Table II our results are compared with those of von Jancsó and von Jancsó, and it is seen that although some of the substances in this group are highly active, the active substances are scattered over a more extensive range of reduction potentials than are the active quinones. This result is perhaps not surprising in view of the fact that the indicators tested have widely different chemical structures and do not, like the quinones, belong to one particular type of compound.



I.



II*a*.



II*b*.

Citrinin (structure I), like the toluquinones we have examined, has a quinonoid structure, and the methyl and hydroxyl groups are present in positions analogous to those occupied by the methyl and methoxyl groups of 4-methoxytoluquinone. Citrinin has, however, a much higher half-wave potential (0·82 v. vs. the S.C.E. at pH 2·03) than any of the toluquinones; in spite of this, its bacteriostatic activity against *Staph. aureus*, 1 in 34,000–50,000, and against *Bact. coli*, 1 in 24,000 (Oxford, 1942*a*), is of the same order as that of fumigatin with a half-wave potential of only − 0·16 v. at pH 6·24.

Penicillic acid (tautomeric mixture of II*a* and II*b*) has against the same strains of *Staph. aureus* an antibacterial potency (1 in 33,000–50,000) of the same order as citrinin, but it is considerably more effective than citrinin against Gram-negative organisms, i.e. 1 in 100,000 against *Bact. coli*. The half-wave potential of penicillic acid is markedly lower, − 0·69 v. at pH 4·63.

DISCUSSION.

The results obtained with the quinones indicate that there is a connection between the oxidation-reduction potentials of a series of closely related substances and their antibacterial potencies. This is not, however, a simple one, other factors obviously influencing the activity adversely, even though the oxidation-reduction potential is within the prescribed limits. The only generalization that we feel justified in making at this stage is that a quinone with an oxidation-reduction potential at pH 7·0 greater than + 0·15 volt or less than − 0·10 volt is unlikely to have antibacterial activity, but it cannot be assumed that all quinones with potentials falling within these limits will have marked activity.

The more variable results obtained with the oxidation-reduction indicators and

with citrinin and penicillic acid indicate the dangers of attempting to apply this generalization to compounds other than true quinones, even when these have a quinonoid structure, as in citrinin and the triphenylmethane dyes. It is evident that oxidation-reduction potential *per se* is not the dominant factor in bringing about inhibition of bacterial growth, as is implied in Dubos' hypothesis that certain dyes are bacteriostatic because they poise the medium at a potential at which the organisms cannot grow. The normal reduction potentials of 4 : 6-dimethoxy-2 : 5-toluquinone and 2 : 5-dihydroxy-benzoquinone, for example, are almost identical, yet the former is strongly antibacterial whilst the latter is relatively inactive. Oxidation-reduction potential, therefore, is probably only one of several physical properties which determine whether a substance is bacteriostatic or not. Other properties which play a part probably include lipoid/water partition coefficient (*cf.* Albert, Goldacre and Rubbo, 1941) adsorption at the bacteria/solution interface, molecular size and shape, and dissociation constant (cf. Albert, Rubbo and Goldacre, 1941 ; Fuller, 1942 ; Bell and Roblin, 1942). It is not unlikely that these properties are interdependent, and that one substance, with a high oxidation-reduction potential and a low dissociation constant, may have the same bacteriostatic potency as another substance with a lower potential and a higher dissociation constant. Only within a series of compounds in which most of the other factors remain reasonably constant will a regular variation in one physical property be accompanied by a regular increase or decrease in bacteriostatic activity.

SUMMARY.

1. The half-wave potentials of phthiocol, phthiocol methyl ether and 2 : 3-dimethyl-1 : 4-naphthaquinone are recorded.

2. The reduction potentials (E'_0) at pH 7·0 of 12 substituted quinones have been calculated from their half-wave potentials.

3. There is no simple relationship connecting the reduction potentials (E'_0) of these 20 substituted quinones with their bacteriostatic activities against *Bact. coli*, but the reduction potentials of all the quinones markedly active against *Staph. aureus* fall between − 0·10 and + 0·15 volt, optimal activity being associated with a potential of about + 0·03 volt.

4. There is no similar relationship connecting the reduction potentials of 18 commercial oxidation-reduction indicators with their activity against *Staph. aureus*. Citrinin and penicillic acid also fail to conform to the generalization made in respect of the quinones.

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THE FIXATION OF FOREIGN MATERIAL IN INFLAMED TISSUE, WITH ESPECIAL REFERENCE TO THE ACTION OF *CL. WELCHII* TOXIN AND ANTITOXIN.

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ONE of the most striking features of antiserum therapy of bacterial intoxication or infectious disease is the disproportionately rapid decrease in the curative effect of antibody with increasing lapse of time between the onset of intoxication or infection and the administration of antiserum. In the later stages of intoxication there is presumably an increasing adsorption of the toxins to regions which, for mechanical or pharmacological reasons, are inaccessible to antibody (see, for example, Buttle and Trevan, 1928 ; Abel and Chalian, 1938). In this connection, the work of Menkin and others (see Menkin, 1940) on the behaviour of foreign proteins in local areas of inflammation must be considered. Menkin has demonstrated that circulating colloidal dyes and foreign proteins accumulate and are " fixed " in local inflammatory areas. According to him, the fixation, which in the course of the inflammation occurs at a time dependent on the nature of the irritant, is maintained by the formation at the periphery of the lesion of a mechanical barrier, consisting of thrombi in the lymphatic capillaries and an intercellular fibrinous network (the " lymphatic blockade "). The barrier prevents both the escape of dyes and proteins from within the lesion, and their access from without.

The possible exclusion of antibody from an inflammatory lesion in this manner has an obvious bearing upon antitoxin therapy, and we have accordingly investigated the fate of antitoxin in lesions produced by the toxin of *Cl. welchii* type A. The response to antitoxin treatment of animals injected with *welchii* toxin is, as in the examples quoted, severely limited by delays in giving antitoxin. In one of a number of tests, each one of which gave similar results, we found that mice receiving an intramuscular dose of *welchii* toxin that would kill them in 6–8 hours could be protected

by an intravenous dose of 0·1 units of antitoxin after 1 hour; after 4 hours an intravenous dose of 40 units was ineffective.

MATERIAL AND METHODS.

Toxin from *Cl. welchii* S. 107 was used throughout; two batches were glycerinated specimens, and one batch a dried specimen. A single batch of proteolytically refined *Cl. welchii* antitoxin containing about 1000 units per ml. was used throughout; it contained low titre anti-hyaluronidase (McClean, 1936) in addition to α-antitoxin. The antitoxin content of sera and tissue extracts was titrated by the Nagler's method against extract of yolk-saline (MacFarlane and Knight, 1941), and an arbitrary toxin standard, chosen so that an initial titre of 1/10 antitoxin was approximately equivalent to one international unit. One volume each of toxin and antitoxin were incubated for ½-hour at 37° C., two volumes of egg-saline added, and the results read after 4 and 20 hours at 37° C.

In our hands the toxin-antitoxin ratio was constant to within a maximum variation of ± 15 per cent. for end-points measured as the highest dilution of antitoxin that totally inhibited opalescence. With low-titre sera and exudates from rabbits, guinea-pigs and mice that had been injected with antitoxin, the toxin-antitoxin ratio was more variable for different standard doses of toxin. The variation is probably due to natural inhibitors of the Nagler reaction present in animal sera. For example, with 10 mouse m.l.d. of toxin (0·7 Lf+/5) as standard in the titration, the average anti-Nagler titres for a number of normal laboratory animals were: 18 rabbits, 1/7·5; 10 guinea-pigs, 1/4·2; 30 mice, 1/6·8. However, titrations made at one sitting with standard toxin proved to be comparable. In no case did we find a measurable amount of natural anti-Nagler substances in extracts of normal tissues.

Tissue extraction.—The tissues were ground in saline with clean sand, 2 ml. of saline to every gramme of tissue, and the fluid separated by spinning. The amount of antitoxin adsorbed to the centrifuged deposit of minced tissue was apparently negligible, for in two tests each of fresh rabbit and guinea-pig normal muscle, 1/3 muscle pastes, shaken at 37° C. for 2 hours with equal volumes of a series of antitoxin dilutions, failed to absorb antitoxin. It is assumed, therefore, that the supernatant fluid from the spun extracts represent a 1/3 dilution of contained antitoxin.

Time relationships of fixation tests.—For convenience in discussing the time relationships of fixation, we refer throughout the paper to two periods, the Period of Induction and Period of Fixation, symbolized by "PI" and "PF" respectively. The period of induction (PI) is the time elapsing between the introduction of the inflammatory agent into the animal, and the introduction into the circulation or tissues of the material —dye or antitoxin—to be fixed. The period of fixation (PF) is the time elapsing between the introduction of the material to be fixed, and the moment either of estimating the degree of fixation or of removing a sample for an estimation. E.g., the symbols PI/PF = 24/1 hours indicates that the fixation of a substance was estimated 1 hour after the injection of the substance into an animal with a 24-hour-old inflammatory lesion.

EXPERIMENTAL.

Fixation of Antibody in Muscle Inflamed by Cl. welchii *Toxin.*

Experiments with rabbits.—Young animals, between 1·6 and 2·0 kg. in weight, were injected half-way along the length of the adductor magnus muscle, with 4–8 mouse m.l.d. of toxin in a volume of 0·25–0·5 ml. The needle was inserted at the

posterior border of the muscle, piercing the skin and gracilis muscle. The animals, excepting where specified, were allowed to move freely in the cage during the PI and PF. From post-mortem examination of over 90 rabbits at various stages of intoxication the relevant sequelae of the injection of toxin may be summarized as follows: Within less than 30 minutes of the injection there is obvious exudation into and round the muscle, with petechial haemorrhages round the site of needle entry. The toxin spreads longitudinally along the muscle, and the severer haemorrhages, exudation and necrosis occur on the proximal half of the injection site, presumably because it is the more dependent part in the sitting rabbit. In a few hours obvious intermuscular and subcutaneous exudate collects, and increases to a maximum at 18–36 hours, by which time there is marked subcutaneous oedema over the whole thigh, with large collections of lightly clotted yellow or faintly blood-stained fluid in the perianal and posterior gluteal regions. After 24–36 hours the subcutaneous and intermuscular pockets of exudate begin to disappear, and are gone by the fourth or fifth day. With the more severe reactions there are perianal and posterior gluteal pockets of exudate on the control side also. On this side the exudate is paler, and even more lightly clotted. This sequence of events takes place irrespective of the injection of antitoxin into the circulation, at any rate with toxin lesions more than 30 minutes old at the time of the injection. After 24 hours, immediately over severely intoxicated muscle, the muscle sheath is opaque, adherent, and flecked with small haemorrhages. The intoxicated muscle consists of mixtures of relatively healthy muscle tissue, obviously distended with fluid, which flows easily from the cut edge, and necrotic tissue, sometimes yellowish, sometimes dark and haemorrhagic. Histological examination confirms the macroscopic appearance. The oedema, characterized by a light deposit of fibrin in the damaged areas, is marked in both healthy and damaged areas, and is maintained up to 120 hours. Moderate numbers of polynuclear cells and a few macrophages appear in the exudate in a few hours; at 24 hours they are collected in the more damaged tissue, and infiltrate an occasional severely damaged muscle bundle. After 24 hours the nuclei of the sarcolemma cells in the necrotic areas are degenerate or missing, and the muscle-fibres of the bundle are fragmented and in some cases partly or wholly absent. The cells in the exudate show signs of degeneration at 24 hours, and at 48 and 72 hours they are represented by pyknotic remnants of their nuclei. At 72 hours fibroblasts appear between the bundles at the edges of the necrotic area. Occasionally a small vein is thrombosed, but at no time up to 120 hours are thrombosed arteries or lymphatic channels visible.

From the appearance of marked exudation to its disappearance on the 4th to 6th day the lymphatic vessels running over the ventral and dorsal surface of the thigh, and those from the skin to the anterior and posterior inguinal lymph nodes, are dilated. The dilatation in some cases was so marked that vessels entering the inguinal nodes were 2 mm. in diameter, and distended with clear lymph that flowed freely for a few seconds after section of the vessel. The lymphatics on the control side were dilated only when the subcutaneous collection of exudates on that side were large, and never to the same extent as those on the intoxicated side.

At the end of the PF the animal was anaesthetized, the circulation stopped, and the heart blood sampled. The skin of the lower limbs was dissected away, each collection of subcutaneous exudate being sampled as it was entered. The flayed lower limbs were then gently wiped free of exudate, and three samples, 0·5 to 1·0 gm., were rapidly cut from both test and control muscles. One weighed sample was extracted for antitoxin titration; a second was coarsely minced, wrapped tightly in a bag of fine muslin, and squeezed until no fluid was detectable on pieces of filter-paper wrapped round the bag during squeezing; the squeezed muscle was re-weighed, to determine the readily

expressible fluid in the muscle. The measurement is obviously crude, but the differences observed were great enough to over-ride the sampling errors. Where possible, samples of expressible fluid were collected for antitoxin titration from the third sample. The antitoxin titres of all samples from one animal were determined in one batch of titrations. The haemoglobin content of the muscle extracts was very low, and never greater than 0·5 per cent. of whole blood, so that less than 0·5 units in the percentage values for antitoxin in the M column of Table I is due to antitoxin contributed by the blood contained in the muscle sample. Antitoxin titres were calculated as percentages of the serum antitoxin titre. The amount of expressible fluid in muscle is conveniently recorded as the weight associated with 100 parts by weight of squeezed muscle tissue; two values, T and C, are obtained for intoxicated and control muscle, and T–C is the weight of *pathological* fluid associated with 100 parts of squeezed intoxicated muscle. The percentage E of pathological fluid in unsqueezed intoxicated muscle is therefore 100 (T–C)/(100+T).

The data from a typical experiment are as follows:

Rabbit 29. PI/PF is 24/6 hours. Toxin in R. leg. Serum antitoxin 1/160.

Antitoxin content (serum = 100).

"Toxin" muscle (extract)	$2·8 = M_t$
,, ,, exudate	$5·0 = E_t$
Control muscle (extract)	$<1 = M_c$
Subcut. exudate, R.	$1·3 = S_t$
,, ,, L.	$<1 = S_c$

Expressible fluid (squeezed muscle = 100).

"Toxin" muscle	$39 = T$
Control muscle	$4 = C$

Percentage "pathological" fluid in "Toxin" muscle:

$E = 100 \times (39 - 4)/(100 + 39) = 25.$

In Table I are summarized the results of 40 experiments. They are arranged primarily in groups (I–IV) of similar PI (period of induction) and within the groups, in order of increasing PF (period of fixation). Taking the data as a whole, it will be seen that in intoxicated muscle the weight of expressible fluid T associated with 100 parts of squeezed muscle ranged widely, from 24 to 126 parts, with a mean of 64·5, and that there is no relation between the total period of intoxication (PI+PF) and the amount of fluid. Oedema is in fact established early in the muscle (see also the T values in Tables II and III), and the only indication of its increase and decrease comes from the size and extent of the observed subcutaneous accumulations, which, as noted above, are maximum at 18–36 hours. The expressible fluid C in control muscle varies from 2–19; the mean is 7·5, with a standard error of ± 5. The percentage of "pathological" expressible fluid, E, in intoxicated muscle varied from 8–52 with a mean of 33.

The antitoxin content of the intoxicated muscle and of different fluids varies with PI and PF; that of the muscle exudate E_t is on the average 1·97 times greater than that in muscle extract, M_t, that of subcutaneous exudate S_t on the "toxin" side less than that in the muscle, and the lowest values for antitoxin content of free fluid in any one animal are those for subcutaneous exudate, S_c, on the control side. The antitoxin content of the control muscle was very low; in 34 of the 40 rabbits it was too small to be demonstrable.

In Group I, in which the PI is 1 hour, the antitoxin content of muscle rises quickly with increasing PF, and that of its exudate is, after 24 and 48 hours, equal to that of the serum. The content of nearby subcutaneous oedema rises equally rapidly, and in Rabbit 4 that of the contralateral oedema was high after 6 hours' PF.

The same general picture holds in Group III, where the modal PI is 24 hours, but here the antitoxin content of the muscle rises much more slowly with increasing PF. In Group I it was 30 per cent. in 2 to 6 hours, in Group III 30 per cent. is first recorded after 24 hours' PF; up to 17 hours' PF the usual figure is about 6, with one value (Rabbit 42) of 14. The same relations hold for Group IV, the development of high antitoxin content of the intoxicated muscle taking 24 hours or more.

These high values associated with a long PF are to some extent fictitious, for the content is expressed in terms of the serum antitoxin titre at the end of the PF. The antitoxin in the circulation is leaking into the muscle from the moment of its intravenous injection, when its concentration is much higher. If the leak from the circulation is continuous throughout the PF, the antitoxic titre of the fluid will be intermediate between the serum titre at the beginning and the end of the PF. In the absence of any knowledge about the rate of exudation during the PF, we cannot decide upon a value for this intermediate titre that would best represent the average antitoxin level in the blood serum that has contributed to the exudate during the PF. The serum titres at the beginning and end of the PF set the limits, and antitoxin contents in the tissues may be calculated only in terms of these two limiting values. Unfortunately the initial titre was not determined in many of our experiments ; but since most of the animals were of approximately the same weight, and all received a standard dose of antitoxin, an estimate of the initial titre can be made in each case from the titre at the end of the PF, using factors derived as follows from a measure of the rate of disappearance of antitoxin from the circulating blood.

In 13 experiments both initial and final titres were determined ; in 8 rabbits the titre dropped in 3–6 hours to an average of 68 per cent. of its original level, in 3 rabbits to an average of 36 per cent. in 24 hours, and in 2 rabbits to an average of 22 per cent. in 45 hours. An indirect measure was obtained from 39 rabbits weighing between 1·6 and 1·8 kg., all receiving precisely 2 ml. antitoxin. The average titres after various periods between injection and sampling of the serum (i.e. PF) were as follows :

PF (hours).	Number of rabbits.	Average antitoxin titre.	Titre as percentage of 1-hour level.
1	16	348	100
2–6	11	250	72
24	6	133	39
48	5	66	20
72	1	20	6

The estimated mean percentage drop in content at 2–6, 24 and 48 hours corresponds reasonably with those obtained by direct observation in the 13 rabbits. An estimate of titre obtaining in the serum 0–1 hours after injection may be made by multiplying the titres in the last column of Table I by 1·4, 2·5, 5·0 or 16·0, according as to whether the recorded PF is 2–6, 24, 48 or 72 hours respectively. In Tables I and II, for experiments with a long PF the estimates calculated on the basis of the initial antitoxin level are recorded in brackets under the data based on final levels.

The corrected values for M_t reach a maximum at 24 hours, after which they remain stationary or decline (all groups). Even allowing the maximum estimated errors both in the determination of E and in the Nagler titrations upon which the value M_t is based, there is no ground for the supposition that concentration of antitoxin in the muscle ever exceeds that found in the serum at some time during the PF.

The picture that emerges from a consideration of the Table, together with the anatomical findings at necropsy, is one of early damage to the blood capillaries, and

TABLE I.—*Showing the Accumulation of Antitoxin-containing Fluid in the Tissues of Rabbits Intoxicated by an Intramuscular Injection of* Cl. welchii *Toxin.*

Rabbit No.	PI (hrs.).	PF (hrs.).	Expressible fluid.			Antitoxin content (serum = 100).					Serum antitoxin (at end PF.).
			T.	C.	E.	M_t.	E_t.	M_c.	S_t.	S_c.	
Group I.											
5	¼	1	nt	nt	..	15	nt	<1	25	nt	480
1	1	½	nt	nt	..	7·5	25	2	10	nt	480
2	1	1	nt	nt	..	7·5	25	2	10	nt	480
6	1	1	nt	nt	..	15	nt	<1	50	nt	240
B	1	1¼	nt	nt	..	7·5	19	1	19	nt	640
A	1	2	nt	nt	..	30	100	<1	50	nt	320
3	1	2	nt	nt	..	20	50	<4	50	..	240
4	1	6	90	10	42	30	50	<4	50	25	240
C	1	24	nt	nt	..	18	100	<2	80	nt	100
						(7·2)	(40)		(32)		(250)
D	1	48	nt	nt	..	60	100	<2	nt	nt	120
						(12)	(20)				(600)
Group II.											
7	2	1	nt	nt	..	7·5	100	<1	50	50	240
8	6	1	nt	nt	..	4	nt	<1	6	nt	480
10	6	17	68	6	37	30	100	<1	1	1	240
19	17	6	92	19	38	7·5	10	<2	7·5	7·5	80
Group III.											
44	24	½	68	3	39	6·5	5·0	<1	1	<½	160
45	24	½	82	2	44	5·6	4·5	<1	1	1	160
K	24	1	nt	nt	..	1·4	3	<1	1	<1	640
36	25	1	65	2	38	3·8	5·0	<1	4	0·3	160
37	25	1	57	3	35	2·5	3·3	<1	2	0·2	240
F	24	1	nt	nt	..	1·5	nt	<1	1·2	1·2	320
40	22	2	33	5	21	6·0	9·0	<½	0·5	0·2	300
41	22	2	53	7	30	6·0	10·0	<½	1·2	0·5	300
42	22	2	69	7	37	14·0	9·0	<1	1·6	2·2	160
43	22	2	93	5	46	2·0	5·7	<½	0·6	1·1	250
38	24	3	38	4	25	4·0	5·0	<½	3	0·5	600
39	24	3	73	7	37	6·5	8·0	<½	4	<½	320
11	24	5	54	9	29	2·8	5	<1	2	..	320
29	24	6	39	4	25	2·8	5	<1	1·3	<1	160
12	30	17	24	14	8	7·5	17	<1	5	25	120
30	24	24	31	7	18	30	40	<3	10	1·4	80
						(12)	(16)		(4)	(0·5)	(200)
31	24	48	124	9	52	60	80	<3	80	nt	40
						(12)	(16)	(<½)	(16)		(200)
32	24	72	58	9	31	60	120	<7	35	nt	20
						(3·8)	(7·5)	(<1)	(2·2)		(320)
Group IV.											
20	40	1	50	8	23	3·8	3·3	<1	1·7	1·7	240
22	44	1	73	8	38	4·2	10	<1	5	5	120
E	48	1	nt	nt	..	6·2	6·2	0·8	0·3	nt	480
21	40	6	126	19	47	10	13	<1	3	5	120
33	48	6	nt	nt	..	11·2	18	<1	5	1	160
34	48	24	29	6	18	60	75	<2	20	5	80
						(24)	(30)	(<½)	(8)	(2)	(200)
G	48	24	nt	nt	..	30	66	<2	13	10	120
						(12)	(20)		(5)	(4)	(300)
35	48	48	60	7	33	90	80	<3	80	40	40
						(20)	(16)	(<1)	(16)	(8)	(200)

For explanation of the symbols see p. 98. nt = no test made.

early exudation, increasing to a maximum at 24 hours. Drainage is inefficient, and fluid collects first in the muscle, and then in the loose tissues surrounding the muscle. The accumulations of exudate spread in the subcutaneous tissue, but after 24 to 48 hours the output of exudate ceases to be in excess of the drainage ; presumably some of the capillaries recover, and after 24 hours absorption exceeds exudation ; after 72 hours only the intoxicated muscle itself contains an appreciable proportion of " pathological " fluid. Thus for the greater part of the time the intoxicated muscle contains a " lake " of exudate. If antitoxin is injected in the circulation soon after intoxication of the muscle, the antitoxin content of the lake rapidly approaches that of the serum (Group I, and Rabbits 7, 8 and 10 of Group II). Antitoxin also leaks into the remoter subcutaneous lakes of exudate. If, on the other hand, antitoxin is introduced into the circulation after the lake of exudate is well established, a much longer period must elapse before the antitoxin level in the lake rises ; any strong antitoxin from the capillaries is diluted in the existing oedema. But the lake of oedema is not stagnant, for relatively high levels are finally established in the fluid, presumably by removal of the existing oedema fluid and its replacement by exudate containing antitoxin.

The observations that abnormal amounts of foreign protein accumulate in inflamed tissues are confirmed. But they do not necessarily support the view that the phenomenon is a " fixation of foreign proteins "—a phrase that implies a special mechanism, which comes into play by virtue of the foreignness of a protein. The " fixation " appears to be no more than the accumulation of exudate, an accumulation that concerns native and foreign proteins equally, at all events if they are of the same size as refined antitoxic horse globulin. If there were fixation in the sense that the tissues held foreign proteins preferentially, it could not be inferred from data such as we have presented, unless the concentrations of the antitoxin in muscle were greater than would be expected from the degree to which the muscle contained an abnormal proportion of fluid. A comparison of the contiguous column E and M_t in the table shows at once that it is not so. The percentage of pathological fluid E in 36 of 40 cases exceeds the percentage of antitoxin (M_t, reckoned as serum antitoxin) found in the muscle. In the four cases where E is less than M_t the PF was 24–72 hours ; and M_t calculated on the basis of initial titre is well below the E value.

Attempts to Alter the Rate of Accumulation of Antitoxin.

The antitoxin concentration in the muscle exudate will not necessarily rise with lengthening PF to a value equal to that in the serum, for leakage will cease from capillaries that recover their normal relative impermeability. The recovery of impermeability after a single dose of toxin may be demonstrated by the intravenous injection of 5 ml. of pontamine blue into a rabbit that has had intradermal injections of an inflammatory but not grossly necrotizing dose of *welchii* toxin on each of the preceding five days. After the dye has been circulating for an hour, the one- and two-day lesions are deeply, and the three-day, moderately stained. The four- and five-day lesions are not stained at all ; that this effect is not due to thrombosis of or stasis in the blood vessels in the lesion, but is due to the recovery of the capillary vessel walls, may be immediately demonstrated by injecting into the site of the lesions a second similar dose of toxin ; the area becomes deeply stained in 5–10 minutes. The rate of recovery will not be uniform, and we might expect, therefore, in muscle in the late stages of intoxication slowly draining areas of the lake into which the inflow has ceased, and areas into which the capillaries are still leaking. If this were so, intravenous injection of antitoxin into an animal with an old lesion should result in immediate exudation of antitoxin into few areas, and an immediate but small rise in the average antitoxin content of the muscle.

TABLE II.—*Showing the Immediate Appearances of Small Amounts of Antitoxin in the Muscle Oedema Fluid of a 2-day-old Intoxication* (*Rabbits* 64–69); *and the Accumulation of Antitoxin-containing Fluid in Turpentine* (*Right Leg*) *and* Staph. aureus (*Left Leg*) *Lesions* (*Rabbits* 55–57).

Rabbit No.	PI (hrs.).	PF (mins.).	Right leg. Expressible fluid. T.	Right leg. Expressible fluid. E.*	Right leg. Antitoxin content. M_t.	Right leg. Antitoxin content. E_t.	Right leg. Antitoxin content. S_t.	Left leg. Expressible fluid. T.	Left leg. Expressible fluid. E.*	Left leg. Antitoxin content. M_t.	Left leg. Antitoxin content. E_t.	Left leg. Antitoxin content. S_t.	Serum titre.
64	48	1·5	143	49	<2	1	0·5	146	54	<1·5	2	0·5	40
65	48	5	141	49	<2	2	1	34	16	<1·5	1	1	80
69	48	6	79	37	<1	4	1	47	23	<1	4	0·5	60
66	48	15	57	28	<1	1·5	<1	80	37	<1	2	<1	60
67	48	60	98	43	<3	nt	<1	22	7	<1	8	1	40
68	48	1440	60	29	19	16	4	73	35	12	16	4	20
					(4)	(6)	(1·6)			(5)	(6)	(1·6)	(50)
		(Hours.)	Turpentine.					*Staphylococcus aureus.*					
55	19	1	21	6·5	7·5	20	6·5	25	9·6	7·5	nt	nt	90
56	46	1	38	18	5·6	10	15	43	21	5·6	17·5	nt	160
57	46	26	132	51	26	30	60	51	25	23	80	40	40
					(11)	(12)	(24)			(9)	(32)	(16)	(100)

* Calculated on the assumption that C = 13.
For explanation of the symbols see p. 98. nt = no test made.

TABLE III.—*Showing the Similar Rates of Accumulation of Antitoxin in the Legs of Anaesthetized Rabbits Intoxicated by Intramuscular Injections of* Cl. welchii *Toxin, the Left Leg being Quiescent, the Right Leg Stimulated Faradically.*

Rabbit No.	PI (hrs.).	PF (hrs.).	Right leg. Expressible fluid. T.	Right leg. Expressible fluid. E.*	Right leg. Antitoxin content. M_t.	Right leg. Antitoxin content. E_t.	Right leg. Antitoxin content. S_t.	Left leg. Expressible fluid. T.	Left leg. Expressible fluid. E*.	Left leg. Antitoxin content. M_t.	Left leg. Antitoxin content. E_t.	Left leg. Antitoxin content. S_t.	Serum.
49	21	¼	88	40	0·9	8	1·2	77	36	0·9	4	1·2	160
48	19	½	72	34	1·9	3·1	0·5	50	25	1·7	2·5	nt	320
46	19	1	39	19	1·8	10	7·5	62	31	5·6	10	5	80
47	20	1½	43	21	6·3	5	1	71	34	3·8	5	1	160
53	19	5	34	16	1	2·5	1	49	24	1·0	2·5	2	160
51	48	¼	93	41	1·8	2·3	1	50	25	1·8	2·5	1	300
50	72	½	87	40	3·7	9	nt	79	37	3·7	9·0	nt	160
52	72	5	58	28	3·7	10	2·2	45	22	3·1	8·2	nt	160

* Calculated on the assumption that C = 13.
For explanation of the symbols see p. 98. nt = no test made.

The hypothesis was tested in 6 rabbits with 48-hour-old bilateral experimental lesions (Table II, Rabbits 64–69). In the absence of a control muscle for determining the normal expressible fluid, the average value of 7·5 plus its standard error (7·5 + 5 = say 13—see col. 5, Table I) was used. The high value was chosen to avoid overestimation of the "pathological" fluid, and a consequent strengthening of the evidence for our general hypothesis that at no time does exudate contain more antitoxin than was present at some time in the parent plasma. Oedema (E column) was well established in all the muscles. Relatively weak doses of antitoxin were used, and measurable antitoxin did not appear in the muscle extract after 1 hour (a 1 in 3 "dilution" of muscle is the highest concentration that can be tested for antitoxin). It was detectable from 1½ minutes onwards in small but gradually increasing amounts, both in muscle exudate and subcutaneous exudate over the muscle. The rate of accumulation of

antitoxin in the exudation Rabbits 66, 67 and 68 may be compared with that in Rabbits 12, 6 and C in Table I. The expected small amounts of antitoxin appear very early in the older lesions, but a large rise in average content takes place only after 20 hours or more.

The marked dilatation of the lymphatic vessels draining the affected parts in the rabbits suggested that drainage was proceeding at a maximum rate—a suggestion supported by the results of unsuccessful attempts to increase the rate of accumulation of antitoxin in muscle by procedures that normally augment the flow of lymph. Five pairs of rabbits, each PI 24 hours and PF varying from ½ to 3 hours, were compared, one quiescent in its cage and one on its back under light anaesthesia on a warm plate. The intoxicated leg of the anaesthetized animal was for the whole PF flexed and extended 30–40 times a minute by a rotating crank. The rate of accumulation of antitoxin was not significantly different in the muscles of quiet and rotated legs. Another test was made on 8 rabbits, intoxicated in both thighs. Under Nembutal anaesthesia one leg was shaved, and at 5–10-minute intervals throughout the PF was given a 5–10 minutes' course of faradic stimuli, continuous, surging, and intermittent, to get maximum alternating contraction and relaxation of the limb. Care was taken that the stimulus did not leak over to the control limb. Table III shows that over a wide variation of PI and a moderate variation of PF there is no significant difference in the rates of accumulation in the right and left legs.

The evidence so far presented is consistent with the simple hypothesis that in *welchii* intoxicated muscle the accumulation of antitoxin (" foreign protein ") is a function of the permeability of blood capillary to antitoxin-containing plasma, and that " fixation " is the result of quantitative incapacity of normal channels to drain away the inflammatory exudate. There is no need to postulate lymphatic blockade.

Fixation with Other Excitants and in Other Sites.

Our experimental conditions differ from Menkin's in the site and the excitant of the inflammation studied. The muscle is poor in lymphatics (Drinker and Yoffey, 1941) and *welchii* toxin, in many respects unlike the pyogenic substances employed by Menkin. We have accordingly tested two of Menkin's agents, turpentine and *Staph. aureus* in rabbit muscle, and these and other inflammatory agents in the dorsal skin of rabbits and the skin of the mouse's ear.

In Rabbits 55, 56 and 57 (Table II) the right leg was injected with 0·2 ml. of a washed suspension of a 20-hour culture of *Staph. aureus*, 10^{10} organisms per ml., and the left leg with 0·1 ml. turpentine. The coccus induces a larger amount of intra- and extra-muscular exudate than turpentine does, but in both types of lesion the relation between PI, PF, E and antitoxin content are essentially similar to those found in *welchii* intoxicated muscle, and in no case does M_t exceed E.

Skin lesions were tested with antitoxin and with pontamine sky blue. A few measures were made of E in skin lesions, and of the antitoxin in the expressible fluid The results with *welchii* toxin, *Staph. aureus* and turpentine are summarized in Table IV. About one mouse m.l.d. of toxin in 0·1 ml. induced a marked lesion which did not proceed to gross necrosis. The excitant was injected intradermally on one side of the midline, and after the lesion was established, a similar area mapped on the other side at the end of the PF ; both were cut out and tested in parallel. With all three excitants, E is on the average at least as high as that in muscle. The lowest value is 7·5, in a 78-hour-old staphylococcal lesion in Rabbit 63, but in all cases there is ample excess fluid to account for abnormal antitoxin content; and where antitoxin in the fluid could be measured, it was never more than 63 per cent. of the serum titre.

TABLE IV.—*Showing the Accumulation of Antitoxin-containing Fluid in Skin Lesions Induced by Various Inflammatory Excitants.*

Rabbit No.	PI (hrs.).	PF (hrs.).	Expressible fluid. T.	Expressible fluid. C.	Expressible fluid. E.	Antitoxin content E_t (serum = 100).	Excitant.
54	1	0	61	21	25	..	*Cl. welchii* toxin.
60	1	½	71	42	17	..	
58	1	1	117	39	36	45	
61	1	3	62	42	12	30	
59	1	23	86	16	38	63	
62	25	3	117	46	32	..	
63	48	23	95	21	38	25	
54	1	0	92	20	38	..	Turpentine.
58	1	1	90	29	32	45	
59	1	23	55	17	25	50	
60	1	½	91	34	25	..	*Staph. aureus.*
63	48	23	45	34	7·5	..	

For explanation of the symbols see p. 98.

That "fixation" need be no more than accumulation of blood plasma that is carrying the foreign material can also be demonstrated by dye tests. Dye was injected intravenously into albino animals with lesions of the desired kind and age in the skin on one side of the animal. A sample of serum was taken from each animal two minutes after the injection. When the lesions had acquired maximum staining, the dye-stained serum, representing the highest concentration of dye attained in the circulating blood, was injected intradermally into the corresponding site on the other side. After the bleb raised by the second injection had subsided—a process taking 30–60 minutes—the intensity of staining in the two areas was compared. Care was taken that the inflammatory agent on the one side and the serum on the other side were injected at similar depths in the skin. A final comparison was made by excising the two skin areas, cutting each of them through the point of maximal staining, laying together half the area from each with the cut edges in juxtaposition, and comparing intensities directly on the upper and under surfaces of the skin. On section there were no macroscopic pockets of dyed serum in the control skin; as in the intoxicated skin, the dye-containing fluid was evidently distributed intimately through the intercellular spaces of the dyed skin.

In a preliminary test two rabbits, about 1·8 kg., were each injected with 0·1 ml. of *welchii* toxin and 0·1 ml. of turpentine, and after a PI 5 minutes were given 2 ml. of 5 per cent. pontamine blue intravenously. The dye-stained serum was injected after 30 minutes and comparisons were made after 1, 2, 4 and 24 hours. The staining of the serum-injected skin was intenser than that in the two lesions at all readings.

More extensive tests were made in the ears of mice. Since it was difficult to obtain large enough samples of the blood from each of the test animals, a control batch was killed 2 minutes after the dye injection, and the blood serum pooled for injection into the opposite ears of the test mice. The following are typical protocols:

Eight mice per batch. PI/PF: 0·5/4 hours. Four batches were injected in the right ear, giving 3·5 mm. blebs of 1 per cent. peptone water, 0·5 per cent. crude *Proteus vulgaris* endotoxin, 48-hour broth culture of *Staph. aureus* and turpentine respectively. At end of PI 0·2 ml. of 5 per cent. intravenous pontamine blue to four mice of each batch and to four control mice, and 0·2 ml. of 0·5 per cent. dye to the remainder of each batch. At the end of the PF the left ears were injected with 3·5 mm. blebs of serum; all mice were killed 30 minutes later, the ears amputated and the blood

samples taken. In no case was the control ear less deeply dyed than the inflamed ear ; often it was more so ; and in each of the animals the concentration of dye in the inflamed area was roughly proportional to that in the serum.

Five mice per batch. PI/PF : 48/2 hours. Procedure as in the previous experiment, using only 5 per cent. pontamine blue. The excitants were 48-hour washed cultures of living *Bact. coli*, *Strep. pyogenes* and *Staph. aureus*, about 10^{10} per ml. in peptone water, all given in the right ear. Here again none of the lesions was as deeply coloured as the serum-injected areas ; indeed, as might be expected with such a long PI, the accumulation of the dye was in some cases poor, especially in the lesions produced by the feebly toxic *Bact. coli*.

Histological examination of the lesions showed well-developed abscesses, 1·0 to 1·5 mm. in diameter, at the centre of areas of staining that varied from 5 to 8 mm. in diameter. There were no signs of marked damage or thrombosis in the lymphatic channels, excepting in the immediate neighbourhood of the small regions of necrosis at the centre of the lesions.

DISCUSSION.

Our measurements of the progressive changes in antibody content of experimentally inflamed tissues are consistent with those of other workers (Fox, 1936 ; Kempf and Nungester, 1939 ; Haviland and McManus, 1941). In Fox's careful work on the time relations of " fixation " the highest recorded concentration of antibody in inflamed tissue was 12·5 per cent. of the serum concentration. Finland (1932), in a study of 19 patients that had received antipneumococcal serum, found only two in which the antibody content of pleural exudate was higher than that in the serum, but the test was apparently made at a time when the level of antibody in the blood would have declined from its initial value. The localization of dyes in inflammatory lesions has been extensively studied (see Burrows, 1932 ; Menkin, 1940), but few of the studies are quantitative. Hirschfelder and others (1924) record in rabbits similar concentrations of mercurochrome in blood and in artificially induced oedema fluid, but no evidence of a *concentration* of dye in the fluid.

The significance of Menkin's hypothesis of lymphatic blockade lies in its relation to defence mechanisms. If " fixation " is to include living bacteria, so that their dissemination is prevented, and antibodies, so that there is local retention of antibacterial substance, the mechanical barrier must surround the area of active inflammation. It is clear that the " powerfully necrotizing irritants " of Menkin will induce inflammation in regions of tissue that subsequently undergo a coagulative necrosis involving small vessels. But the region of an acute infective inflammation includes as well a zone of surrounding tissues in which the permeability of the blood capillaries is grossly increased by the action of toxins, tissues in many cases already invaded by bacteria. We may define the extent of the inflammatory lesion in its early stages in terms of ready staining by a circulating electronegative dye like trypan blue—a definition that is implicit in Menkin's work on inflammatory fixation. In our own work we have, at the periphery of inflammatory lesions so defined, found no evidence either of an effective fibrinous barrier, or of the lymphatic thrombosis postulated by Menkin as the prime cause of retention of accumulated exudate. It is true that we were unable to demonstrate in these lesions the existence of unimpeded lymphatic drainage, by measures designed to increase the outflow of lymph and consequently the inflow of antibody, but in these cases it appeared that the lymphatic drainage was already working to its full capacity.

Our mice and rabbits displayed all the phenomena that have been interpreted

as " fixation " or " concentration " of foreign material in inflammatory lesions. But, in our view, there is no good reason for assuming that this fixation is in the first place any more than an accumulation of exudate containing the foreign material, and in the second place, a retention in the tissue spaces due to an excess of inflow through the walls of damaged blood capillaries over outflow through patent lymphatic channels. Since damage to the lymphatic endothelium increases its permeability, probably to a greater extent than similar damage increases that of the blood capillary endothelium (Hudack and McMaster, 1932), the lymphatic capillary wall will as readily permit the outflow of large protein molecules as the damaged blood capillaries permitted their inflow to the tissues. And, as the experiments of Field, Drinker and White (1932) show, inflammation of a degree not likely to lead to necrosis enhances the flow of lymph.

Rich (1936) questions the importance of the fibrin barrier in " fixation," suggesting that dyes and proteins are absorbed on to the precipitated proteins in inflammatory lesions. Absorption will depend on the nature of the inflammation and of the substance fixed (Burrows, 1932 ; Miller, 1938) ; but though in some cases it undoubtedly occurs, our results with *welchii* antitoxin clearly show that there is no need to assign to it the major role in the retention of foreign substances.

There was no unequivocal indication of capillary thrombosis in stained sections of *welchii*-intoxicated muscle. It is, however, difficult to distinguish in stained sections between a lymph vessel occluded by a recent thrombus and a dilated lymph vessel whose contents have been clotted during the preparation of the tissue for section. But Miles(1943), in a study of the effect of toxins and pyogenic bacteria on the lymphatics in the mouse's ear, has demonstrated that, excepting for tissues in the centre of the lesion that are destined to complete necrosis, lymphatic channels remain patent over the whole inflammatory area.

The lesions produced by *welchii* toxin in rabbit muscle were apparently little affected by intravenous antitoxin given even as early as 30 minutes after intoxication, which suggests that the major damage occurs soon after the application of toxin to the tissues. If this holds also for natural infection, the early introduction of antitoxin into the blood will result in its exudation into damaged tissues, and a neutralization of free toxin in the intercellular spaces. But if there has already been exudation, antitoxin will escape with difficulty, or not at all, from damaged capillaries, and its chief value will lie in protecting tissues not yet intoxicated. Presumably, as soon as the toxin affects healthy tissue, capillary damage results in an exudate of antitoxin. But this simple exudative response might be ineffectual, if excess exudate from a nearby lesion flows into the healthy tissues ; when toxin diffuses into such tissues, the exudation of antitoxin-containing plasma from the newly damaged capillaries will be reduced or inhibited by reason of the high hydrodynamic pressure of the already established overflow exudate.

The inaccessibility of toxin to circulating antitoxin appears to be due in part to the exuberance of exudation it induces—an exuberance that successfully nullifies any benefit to be derived from the convenience, teleologically considered, of the ready escape of circulating antibody into the intercellular spaces of tissues already attacked. If this hypothesis is correct, any attempt to minimize the formation of exudate by, say, the application of a tight plaster to the infected limb, though it would reduce the volume of the " lake " in which antitoxin exuding from the circulation might be diluted, would not materially improve the supply of antitoxin to the infected part, since the osmotic and hydrostatic state of the exudate would still be disadvantageous to free exudation, and, according to the experiments of Barnes and Trueta (1941), the lymphatic drainage reduced to a minimum.

SUMMARY AND CONCLUSIONS.

Exudate derived from the circulation accumulates in the intercellular spaces of muscle or skin inflamed by *Cl. welchii* toxin, and is retained there mainly because of the quantitative inadequacy of existing lymphatic drainage. If the circulation contains antitoxin in the early stages of the inflammation, the accumulating exudate will be relatively rich in antitoxin. Antitoxin introduced in the later stages passes only slowly into the lesion, by reason of the already accumulated exudate, thus accounting, at any rate in part, for the relative inefficacy of antitoxin administered in the later stages of a local progressive *Cl. welchii* intoxication.

In the course of artificial *welchii* intoxication there is no evidence that the accumulation of antitoxin is necessarily a " fixation " or a " concentration " of foreign protein ; neither pontamine blue nor proteolytically refined horse antitoxic globulin appeared in the lesions in concentrations greater than could be accounted for by the simple exudation of the blood plasma into which they were originally introduced ; nor is there evidence that the retention of antitoxin is due to lymphatic blockage, or the formation of a fibrinous network around the lesion.

The accumulation and retention of exudate, and the damming back of further exudate in spite of persisting permeability of the capillary endothelium, afford a sufficient explanation of " fixation " and its consequences, as revealed by the techniques hitherto employed, but it is not claimed that the explanation is necessarily complete. Moreover, though the simple hypothesis may prove applicable to the " fixation " of a wide variety of foreign materials in many types of inflammatory lesion, no ready generalization is possible ; the result in each case will depend upon the chemical and physical properties of the different foreign substances and of the different inflammatory lesions.

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HELVOLIC ACID, AN ANTIBIOTIC PRODUCED BY *ASPERGILLUS FUMIGATUS, MUT. HELVOLA* YUILL.

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WILKINS and Harris (1942) reported that culture filtrates of *Aspergillus fumigatus, mut. helvola* Yuill, showed antibacterial activity. In the following communication the isolation in the crystalline state of an antibacterial substance produced by this fungus, and some of its chemical and biological properties, are described. The substance has been named helvolic acid.

Production and Chemical Properties.

Culture medium and conditions of growth.—The culture of the fungus was supplied by Dr. Wilkins, and for the production of helvolic acid it was grown throughout on the medium suggested by Wilkins and Harris, slightly modified :

Dextrose	40 g.	$MgSO_4$	0·5 g.
$NaNO_3$	3 g.	$FeSO_4$	0·01 g.
Na_2HPO_4	1 g.	Water	1000 ml.
KCl	0·5 g.	Adjust if necessary to pH 8 with NaOH.	

The fungus was grown at 24° C. in porcelain vessels (Abraham *et al.*, 1941) which each contained 1·2 l. of culture medium. The antibacterial activity of the culture was tested against staphylococci by the " ring " method (Abraham *et al.*, 1941). A maximum ring of inhibition, 16–18 mm. in diameter, which coincided with a pH of about 6·8, was reached in from 7 days onwards, the batches varying in the rate at which they matured. The vessels were usually harvested after two or three weeks' incubation.

Isolation of helvolic acid.—The culture medium, which was handled in batches of 100 l., was freed from mycelium by filtration through cotton-wool on large Buchner funnels ; 0·5 per cent. by weight of activated charcoal (Farnell's No. 14) was added and the pH adjusted to 4. The helvolic acid, which is only very slightly soluble in water at this pH, was adsorbed quantitatively on the charcoal. The charcoal was collected on large Buchner funnels and slowly percolated with 80 per cent. acetone* of which 1/5th of the volume of culture medium was needed for complete elution. The acetone was removed by vacuum distillation at + 50° C., the condenser and receivers being cooled efficiently to recover a good yield of the solvent. When most of the acetone had been removed the active substance, together with inactive impurities, precipitated. It was then dissolved in chloroform and purified by chromatographic adsorption on magnesium oxide (Harrington " heavy "). For the purification 4·5 gr. crude helvolic acid was dissolved in 25–30 c.c. of chloroform and passed through an

* We are indebted to Dr. C. G. Pope and Dr. M. F. Stevens of the Wellcome Physiological Research Laboratories for information on the adsorptive qualities of this charcoal and the elution with acetone.

adsorption column 3 cm. wide and 20 cm. long. Helvolic acid is colourless and non-fluorescent in ultra-violet light, while most of the impurities show fluorescent bands. The column was developed with chloroform containing 10 per cent. methyl alcohol until a blue fluorescent band visible at the top of the column was just about to be washed through. To locate helvolic acid, which was adsorbed in the middle fraction of the column, the column was divided up into segments and small portions of magnesia from each were eluted with 1 c.c. of water for 5 minutes. The centrifuged eluates were brought to pH4, and the precipitates at this pH corresponded in amount with the helvolic acid present. The parts of the column giving the largest precipitates were combined and eluted by shaking with successive portions of boiling water until acidification of the eluate gave only a slight precipitate, when the whole eluate was acidified to pH 4. After keeping overnight at + 2° C. the precipitate was collected and dissolved in acetone, and water was added until a faint permanent precipitate was obtained. On standing overnight at + 2° C. the helvolic acid crystallized out in fine needles. For further purification it was recrystallized several times from acetone-water. The yield of helvolic acid averaged 0·4 g. from 100 l. of culture fluid.

Elementary analysis, molecular and equivalent weights.—Helvolic acid contains no elements except carbon, hydrogen and oxygen. The following figures for C and H (Weiler and Strauss) were obtained with different samples of helvolic acid after drying *in vacuo* at 100° C. :

	C.	H.
1	69·4	7·9
2	69·0	7·8
3	69·3	7·7

The molecular weight determined from crystallographic X-ray data (Crowfoot and Low, 1943) was 562 ± 12 ; by the Rast method in camphor (Weiler and Strauss) it was 510. On titration with N/10 sodium hydroxide 1·027 mg. of helvolic acid used up 18·3 µl. of alkali, corresponding to an equivalent weight of 562. Taking into account the figures for molecular and equivalent weights and the analytical data, the most probable empirical formula for helvolic acid is $C_{32}H_{44}O_8$ (calc. C : 69·1, H : 7·7 ; molecular weight 556). The determination of active hydrogen by the Zerewitinoff method in pyridine gave a value of 0·53 per cent. of active hydrogen, corresponding to three active hydrogens per molecule (calc. 0·54 per cent.).

Melting-point.—The melting-point of helvolic acid determined in the capillary tube is 204·5°–205·5° when recrystallized from pyridine, and 209° when recrystallized from 70 per cent. acetic acid. The melting-point determined by a micro method on a single crystal recrystallized from either of those solvents was 212°.

Optical activity.—Helvolic acid is laevorotatory. A 3·5 per cent. solution in chloroform gave an $[\alpha]_D^{20}$ of − 49·4°. A 2·1 per cent. solution in water (as sodium salt) gave an $[\alpha]_D^{20}$ of − 47·3°.

Solubility.—Helvolic acid is practically insoluble in water and easily soluble in acetone, warm alcohol, chloroform, glacial acetic acid and pyridine ; it is moderately soluble in ether and benzene and is very slightly soluble in petrol. The sodium salt of helvolic acid is fairly soluble in water, but the calcium, magnesium, barium, iron, copper, zinc and mercury salts are very sparingly soluble in water.

Ferric chloride gives a yellow precipitate but no characteristic colour reaction.

Reducing power.—Helvolic acid does not reduce Fehling's solution on boiling, nor does it reduce ammoniacal silver nitrate.

Stability.—The antibacterial activity of helvolic acid is not affected by boiling in 2N acid, at neutral pH or at pH 10 for 15 minutes, but boiling in N alkali for 15 minutes and incubation in N alkali at 37° C. for 5 hours destroys the antibacterial activity. No loss of activity occurred after incubating suspensions of the copper and zinc salts respectively in solutions of copper and zinc sulphate for 16 hours at 37° C. Free helvolic acid could be regenerated from the salts by acidification, extraction with chloroform and re-extraction into water with dilute alkali.

Action of diazomethane.—Helvolic acid is converted into its methylester with diazomethane. To 10 mg. of helvolic acid in 1 c.c. chloroform cooled to — 30° C. was added 0·4–0·5 c.c. of an ethereal solution of diazomethane containing approximately 20 mg. per c.c., also cooled to — 30° C. The mixture was allowed to warm up slowly to + 2° C. in the ice chest, where it was kept for several hours. The solvent was then evaporated *in vacuo* and the crystalline residue was recrystallized from a benzene-petrol ether mixture or from methyl alcohol. Different samples of recrystallized methylester had melting-points of 261° and 257° C.

The analysis of different samples gave the following figures :

	C.	H.	OCH_3.	
1	70·6	8·3	5·46	dried at room temperature.
2	69·1	7·9	—	dried *in vacuo* at 100° C.
3	70·3	7·7	5·0	dried *in vacuo* at 100° C.

The determination of the molecular weight of the methylester from crystallographic X-ray data gave a value of 541 ± 11. The analytical data fit fairly well the empirical formula of $C_{33}H_{46}O_8$ (calc. C : 69·5 per cent. ; H : 8·1 per cent. ; OCH_3 : 5·44 per cent.), which agrees with the molecular weight found by X-ray analysis.

Antibacterial Action.

Dilution tests.—The sensitivity of a number of bacterial species was tested by the dilution method.

Technique.—Tubes of 5 c.c. of heart extract broth containing serial dilutions (two-fold throughout these experiments) of sodium helvolate were inoculated with a uniform amount of bacterial culture. For streptococcus, meningococcus and *Cl. septique* 10 per cent. of serum was included in the medium. For all the anaerobes glucose broth was used. Before addition to the medium the helvolate solution was generally sterilized by glass filtration, but if it was very concentrated (1 : 50) this step was omitted, as strong solutions tended to crystallize out after filtration. In this case the solution was made up with careful precautions against contamination. Inoculation was made with a Dreyer's pipette from an 18 to 20-hour culture in broth, serum broth or, with the anaerobes, cooked meat medium. With hardy organisms the culture was often diluted. An early experiment showed that the size of inoculum would modify the result of the test. Since bacterial counts which would equate one species accurately with another were not done the comparison of sensitivity between different species is only an approximation. Some species were tested with two or three inocula differing a thousandfold, to give an idea of the order of variation. To emphasize this point the table has been arranged by groups and not by order of sensitivity. Results were read after overnight incubation by naked-eye comparison of the opacity of the tubes.

Results.—Table I shows that sodium helvolate inhibits the growth of Gram-positive organisms, including those causing gas gangrene, but is almost inactive against the Gram-negative organisms with the exception of the meningococcus and *S. Gaertneri.*

TABLE I.

Organism.	Inoculum of 18 to 20-hour liquid culture.	Dilution of sodium helvolate giving inhibition.		
		Complete.	Partial.	None.
Staph. aureus	1 drop	80,000	160,000 to 1,280,000	2,560,000
	1 drop 1 : 1000	1,280,000	2,560,000*	..
	1 drop 1 : 1,000,000	Above 2,560,000*	..	..
Staph. aureus; bovine strain used for therapeutic experiments	1 drop 1 : 1000	1,280,000	2,560,000*	..
Strept. pyogenes	1 drop	40,000	80,000 to 640,000	1,280,000
	1 drop 1 : 1000	640,000	1,280,000*	..
Strept. viridans (from bacterial endocarditis)	5 drops	..	10,000† to 160,000 slight 320,000 to 640,000	1,280,000
	1 drop 1 : 200	160,000	320,000 to 640,000	1,280,000
Strept. pneumoniae, Type III	1 drop	10,000	20,000 to 40,000	80,000
	1 drop 1 : 1000	160,000	320,000 to 640,000	1,280,000
B. anthracis	1 drop	160,000	320,000	640,000
	1 drop 1 : 1000	320,000	640,000 to 2,560,000*	..
C. diphtheriae gravis	1 drop	640,000	1,280,000 to 10,240,000	20,480,000
Cl. welchii	1 drop	320,000	640,000 to 1,280,000	2,560,000
Cl. oedematiens	2 drops	320,000	640,000 to 2,560,000	5,120,000
Cl. septique	.2 drops	80,000	160,000	320,000
N. meningitidis	5 drops	40,000	80,000 to 160,000	320,000
S. typhi	1 drop 1 : 1000	500	1,000	2,000
S. Gaertneri	1 drop 1 : 1000	25,000	50,000	100,000
Bact. Flexneri Z.	1 drop 1 : 1000	..	..	25,000†
Bact. Shigae	1 drop 1 : 1000	..	..	25,000†
Bact. Sonnei	1 drop 1 : 1000	..	..	25,000†
Bact. coli	1 drop 1 : 1000	500	1,000	2,000
Proteus	1 drop 1 : 1000	500	1,000 to 4,000	8,000
Ps. pyocyanea	1 drop 1 : 1000	500	1,000 to 4,000	8,000
M. smegmatis	4 drops	..	500† to 8,000	16,000

* Highest dilution tested. † Lowest dilution tested.

This range of activity is very similar to that of penicillin. While exerting a powerful inhibition as measured against the sulphonamides, it is very much less potent that the purest penicillin available. The number of organisms present in the inoculum exerts an influence on its activity, as is also the case with the sulphonamides, but in contrast with them its antibacterial action is not inhibited by peptone. There was no striking change in the morphology of partially inhibited cultures.

Action on bacteria.—From the following experiments it appears that sodium helvolate is chiefly a bacteriostatic, not a bactericide. (1) The oxygen uptake of staphylococcal suspensions, measured in Warburg vessels (temp. 37° C., oxygen) was not affected by a concentration of 1 : 1000 over a period of 3 hours. (2) Equal parts of a staphylococcal culture and of a 1 in 500 solution of sodium helvolate were mixed and incubated together overnight. A loopful subcultured into broth produced a full growth of staphylococci. Here, however, the very large number of organisms present might be expected to diminish the effectiveness of the sodium helvolate. (3) At the completion of a dilution test against staphylococci, in which there was no visible growth below 1 : 640,000, a loopful from every tube was plated out. Colonies grew out from every tube down to 1 : 40,000, the lowest dilution tested.

Development of resistance by bacteria.

(*a*) *In vitro.*—Sensitive strains of streptococcus and staphylococcus were subcultured daily in broth containing a partially inhibitory concentration of sodium helvolate. It was clear after a few days that resistance was developing, and the concentration of helvolate was repeatedly increased. After 26 days of passage both the

organisms required a concentration of helvolate 250 times greater than the original strains required to inhibit growth completely. Their sensitivity to penicillin had not decreased.

(b) *In vivo.*—In the 5th therapeutic experiment the infecting strain of staphylococcus was recovered from two mice which died on the 6th day. Each mouse had received 80 mg. of helvolate in 5 days. The titres of the original and recovered strains were the same.

TABLE II.—*Effect of Biological Fluids, by Plate and Cylinder Ring Test.*

Fluid tested.	Final dilution of helvolic acid (as sodium salt).	Incubation of mixture before testing (hours).	Ring size, mm.; test fluid present.	Ring size, mm.; control.
Serum	1 : 1,000*	No incubation	20·0	24·0
	1 : 1,100	,,	20·9	21·9
Pus	1 : 10,000	,,	22	25
	1 : 20,000	,,	20	22
Yeast extract	1 : 8,000	,,	19	22
	1 : 16,000	,,	18	20·5
Bacterial extract	1 : 1,500	,,	22·5	23·5
	1 : 1,000	,,	†(*a*) 29	30
		,,	(*b*) 29	31
	1 : 5,000	,,	(*a*) 24	25·5
		,,	(*b*) 26	26
	1 : 25,000	,,	(*a*) 22	22
		,,	(*b*) 21	22
	1 : 125,000	,,	(*a*) 17·5	18
		,,	(*b*) 18	18
	1 : 625,000	,,	(*a*) 13‡	15‡
		,,	(*b*) 14‡	15‡
Para-amino-benzoic acid 1 : 200	1 : 1,000*	,,	(*a*) 24	25
		,,	(*b*) 24	24
Liver extract	1 : 2,000*	2	25·0	27·0
		17	25·0	28·0
Whole blood	1 : 2,000	2	(*a*) 18	
			(*b*) 20	
		4	(*a*) 19	21
			(*b*) 21	23
		6	(*a*) 19	
			(*b*) 22	

* An impure preparation of helvolic acid was used.
† (*a*) and (*b*) are readings from duplicate plates.
‡ Partial inhibition only.

Effect of biological fluids (Table II).—Various biological fluids and cell extracts were tested to see whether they interfered with the antibacterial action. The "ring" method was employed. Sodium helvolate in aqueous solution was mixed with the test fluid, which constituted at least 50 per cent. of the final mixture. Occasionally the mixture was incubated at 37° C. before testing, but usually it was put directly into the test cylinders and interaction of the fluids and diffusion into the agar allowed to proceed together while the plate was being incubated. In testing whole blood the mixture was rotated in the incubator to keep the cells moving, and cell-free samples

were withdrawn for testing at 2, 4 and 6 hours. Pus presented some difficulty, as its high viscosity prevented complete emptying of the cylinders and so, possibly, prevented complete diffusion. After several attempts with whole pus the supernatant fluid from a sample of pus was used instead ; this is the experiment shown in the table. The bacterial and yeast extracts were prepared by grinding in a bacterial mill (Booth and Green, 1938) ; the breaking up of the cells was very satisfactory. All fluids reduced the ring size slightly, the bacterial extract the least. The reduction could be considered as equivalent in some cases to a loss of about 50 per cent. of the activity of the sodium helvolate, but it is quite possible that the effect is partly due to slower diffusion of the active substance from viscous fluids. With bacterial extract, para-aminobenzoic acid and whole blood the effect is slight or negligible. In a serial dilution test against the staphylococcus 10 per cent. of serum added to the medium in no way diminished the inhibitory power, and so, too, with 50 per cent. of filtered broth in which a staphylococcal culture had grown for 5 days at 37° C.

Absorption and Excretion and Pharmacological Effects.

It may be surmised that any substance which is active against bacteria *in vitro* will be found to be largely excreted in the urine, for otherwise it would probably be fixed to the tissues and be either toxic or ineffective.

The excretion of helvolic acid was followed in 20 gm. mice. (Mice of about 20 gm. have been used throughout.) The urine secreted during the 6 hours after an intravenous injection of 3 mg. gave a ring of inhibition 25 mm. in diameter, after 2 mg. a ring of 23, after 1 mg. a ring of 20, and after 0·5 mg. a ring of 12. Sodium helvolate is therefore excreted by the kidneys unchanged or in a still active form.

Experiments were also made to find out whether a significant level of antibacterial activity could be maintained in the blood. In one experiment 10 mice were given 1 mg. of sodium helvolate in 0·1 c.c. intravenously. One mouse was killed every hour and a blood sample obtained by cutting the inferior vena cava. Serum from the sample taken at 1 hour gave a clear ring of 17 mm., that at 2 hours a clear ring of 15 mm., and thereafter to the 6th hour there was just discernible inhibition. Beyond this time no inhibition could be seen. After a 3-mg. dose, however, there was a clear ring of 13 mm. at the end of 10 hours, and this was of particular interest as the urine in the bladder only gave a ring of 11 mm., which suggested that the material was not being excreted very fast. These mice appeared perfectly well up to the time they were killed.

These 10 mice also gave an opportunity to confirm an observation made previously on 3 mice. The liver, spleen and kidneys were excised from each animal, ground up with a little saline and tested. The liver extract gave marked inhibition in every mouse but one, while spleen and kidney extracts were inactive except for slight inhibition in the mouse killed at 1 hour. This result strongly suggested that the sodium helvolate was being excreted by the liver rather than stored in the reticulo-endothelial system, for in that case inhibition by the spleen extract would be expected.

Utilizing the fact that the substance was excreted by the kidneys, there was no difficulty in showing that it was readily absorbed from the peritoneal cavity and from the subcutaneous tissues ; again the livers had a high antibacterial content. After 3 mg. by stomach-tube, however, only a barely discernible inhibition was present in the urine. This result might have been due to the non-absorption of the material or to its destruction in the gut. To decide the point two mice were given 10 mg. in 0·3 c.c. water by stomach-tube and were killed at the end of 7½ and 8 hours respectively. The serum gave rings of 16 and 18 mm., the livers rings of 16 and 17 mm. The urine

secreted during the last 5 hours of experiment was obtained from one animal and gave a ring of 17 mm., about the same as the blood, again suggesting that the active substance was not excreted very fast by the kidneys. The stomach, small intestine and large intestine were excised after dividing between ligatures. The contents were separately washed out with water and the fluid, free from gross débris, put into cylinders for testing. Rings of 20 and 26 for the stomach contents, 20 and 23 for the small intestine and 22 and 23 for the large intestine were obtained. It was thus clear that the material was absorbed slowly over a long period and there was no evidence of serious destruction in the gut. Enough sodium helvolate was absorbed from a dose of 10 mg. to give a good inhibitory level in the blood, and active material was present at the end of 8 hours throughout the gastro-intestinal tract, so that it may be assumed that the blood level would be maintained still longer. The animals appeared quite well during this experiment.

Experiment on a cat.—The object of this experiment was to observe the effect of an intravenous injection on blood pressure and respiration, to determine whether the material was secreted in the bile and to follow the blood levels.

The cat (wt. 3·2 kg.) was anaesthetized with chloralose and a continuous blood-pressure record was made from the carotid artery. The bile duct and urethra were cannulated. Sodium helvolate, 150 mg. in 12 c.c. water injected into the internal saphenous vein, caused a momentary fall of blood pressure at the beginning of the injection (from 164 mm. Hg to 140 mm.), but by the end of the injection, which took 3½ minutes, the pressure had risen to 150 mm. In another 6 minutes the pressure was again 164 mm. There was no appreciable effect on the depth or rate of respiration or on the heart rate. Samples of bile were collected every half-hour for 4 hours, the rate of secretion being about 2·5 c.c. an hour. Every sample, when assayed by plate and cylinder, showed very strong inhibition (rings of 22 to 26 mm.). Thus the opinion formed from the mouse experiments that the active material was, like penicillin, secreted in the bile was confirmed.

During the whole of the experiment a good inhibitory level was present in the blood (rings of 19 to 14 mm.). Only 8·8 c.c. of urine was secreted in the 4 hours of the experiment; this gave a ring of 18 mm. While it would not be justifiable to lay much stress on urinary secretion in an anaesthetized animal, this result again suggests that the substance is not very readily excreted in the urine. The cerebrospinal fluid, which was collected at the end of the experiment, gave no sign of inhibition. Some pericardial fluid gave a ring of 15 mm. It may be inferred that the inhibitory substance does not pass freely into the normal cerebrospinal fluid, but does pass out of the blood into the tissue fluids.

Toxicity.

Local administration.

Leucocytes.—The method previously described (Abraham *et al.*, 1941) was used. Two experiments agreed in showing that a 1 per cent. solution of sodium helvolate (which was very hypertonic) killed most of the leucocytes immediately and all within an hour. In a 1 in 800 solution the cells were sluggish from the beginning and many were dead at 3 hours. In 1 in 1600 the cells did not differ appreciably from the controls. A solution of 1 in 1600 or over has, therefore, under the conditions of these experiments, no substantial action on polymorph leucocytes. Comparison of this figure with the figures for bacterial inhibition shows a wide gap between the dilution which will inhibit a sensitive organism and that which will injure leucocytes.

Cells in tissue culture.—We are indebted to Dr. P. B. Medawar, of the Department

of Zoology, for carrying out these tests. The concentration of the drug just suppressing outgrowth from the explant for 24 hours was approximately 1 : 1500. In this concentration the cells underwent vacuolar degeneration. The highest concentration the tissues tolerated for 48 hours without undergoing irreversible changes was 1 : 2500. Tissues from this concentration grew out when subcultured into normal medium at the end of the 48 hours. Here again it is clear that tissue cells will withstand very much more concentrated solutions than susceptible bacteria.

Central nervous system.—The effect on the tissues of the central nervous sytem was studied by injecting sodium helvolate solution into the cisternal space of rabbits. Under ether anaesthesia a needle was inserted and some cerebro-spinal fluid withdrawn. In two rabbits 0·5 c.c. of a 1/1000 solution of sodium helvolate was injected. On recovery from the anaesthetic these rabbits showed no sign of damage or irritation. When the cisternal space was tapped again 18 hours later antibacterial activity could be detected in one cerebrospinal fluid but not in the other.

A similar injection of 1/250 solution into two rabbits produced definite signs of irritation—muscular spasm and weakness and, in one rabbit, fits—lasting about an hour, after which recovery appeared to be complete. Both cerebrospinal fluids showed a trace of activity 24 hours later.

Skin and subcutaneous tissues.—Injections of 1/100 and 1/1000 sodium helvolate into the ear of an albino rabbit caused some hyperaemia, but otherwise no damage could be seen.

Mice receiving subcutaneous injections of 5 per cent. solution developed some oedema at the place of injection, to which the hypertonicity of the solution may have contributed. At the site of repeated subcutaneous injections of a 1/50 solution there was histological evidence of a fibroblastic reaction but no necrosis.

Peritoneum.—Three mice received 4 mg. in 0·2 c.c. (a 1/50 solution) on 6 successive days by intraperitoneal injection. On the 6th and 7th days respectively two of the mice fell sick and were killed, and the third, which still appeared well, was killed on the 8th day. All the mice had advanced aseptic plastic peritonitis, with superficial necrosis of the liver, and in the third, which lived the longest, the deeper parts of the liver showed areas where there was vacuolation of the cells.

General administration.

Immediate effect.—The difficulty of producing the substance in sufficient amounts prevented the estimation of the lethal dose on a large enough number of mice to be of statistical importance. A few mice, however, were given large doses, and from these it can be stated that 10 mg. of sodium helvolate dissolved in 0·2 c.c. of distilled water given intravenously causes the death of a 20 gm. mouse within an hour, and usually within a few minutes. A mouse will recover after an injection of 5 mg., though it may appear sick for a short time, and a dose of less than 5 mg. is tolerated without apparent disturbance. The reason for the immediate lethal effect of the larger doses is not clear, but in view of the relative insolubility of calcium helvolate it is possible that interference with calcium distribution may play a part. On subcutaneous injection, 10 mg. killed but 5 mg. did not. A dose as large as 20 mg. in 0·3 c.c. by stomach-tube produced no apparent disturbance.

Late effects.—One mouse out of two which received 7 doses of 10 mg. in 4 days by stomach-tube died on the 12th day ; the other, and also one which had 9 doses of 10 mg. in 6 days, were apparently well on the 14th and 16th days respectively and survived. These animals were not investigated histologically, but when the last mouse-protection experiment was done the opportunity was taken to give two mice the full

course of drug treatment, 2 mg. twice daily for 5 days by intravenous or subcutaneous injection, without the streptococcal infection, and to examine them more fully. The mice remained lively during and after the course of injections, and though their coats were less sleek than the normal there was nothing in their appearance or behaviour to suggest any serious deleterious effect. They were killed respectively on the 5th day (mouse D) and 16th day (mouse E) and histological examination made. The lungs, stomach, small and large intestines and adrenal were normal. The bone-marrow and the lymph follicles in the lymph glands and spleen were full of cells and appeared to be in a normal state of activity. The kidneys in E were normal, but in D there was here and there flattening and raggedness of the cells of the proximal convoluted tubules—possibly a sign of early damage. In contrast with the picture in other organs, the livers of both animals showed severe damage. The cytoplasm of the liver cells was vacuolated in many areas to such an extent that only a rim was left around the well-defined borders of the cells. An early stage of this extreme change appeared to be the formation of a fine foamy structure in the cytoplasm. The nuclei of the cells appeared to be normal. The liver of mouse E, killed 11 days after the last injection, was indistinguishable from that of the animal killed at the end of injection. It would thus appear from histological observations that helvolic acid given repeatedly is poisonous to the liver, which undergoes marked changes, while the kidneys through which the substance is also excreted show little if any change.

Mouse Protection Experiments.

When these experiments were undertaken it appeared that a concentration of sodium helvolate which will inhibit the growth of bacteria in the relatively insensitive ring test could be obtained in the blood of animals without any immediately toxic effect. The liver damage was only detected in the course of the observations about to be reported. The experiments were done to show whether the substance would protect mice against a lethal dose of organisms given intraperitoneally.

Method.—Mice weighing about 20 gm. were used throughout. The infecting organism was given intraperitoneally. The staphylococcus was a bovine strain, Wellcome 133, particularly virulent to mice. The dose was 0·5 c.c. of a 16 to 20-hour broth culture, opacity equivalent to about 335 million organisms per dose. The streptococcus was also supplied by the Wellcome Laboratories and was a Richards strain, C.N. 11, of which the virulence rose rapidly on passage. The dose was 0·5 c.c. of a 1 in 10,000 dilution of a 14-hour serum broth culture. In two out of three preceding passages 0·5 c.c. of a 1 in 100,000,000 dilution of a similar culture had killed by the third day. Treatment was started an hour after the infection and was generally repeated 12-hourly.

Results (Table III).—Various routes and dosages were employed in an attempt to produce survival after the staphylococcal infection, but only prolongation of life could be shown. At post-mortem on mice dying early the staphylococcus was recovered in pure culture from the heart blood and peritoneal fluid. Those dying later had abscesses in the omentum and liver and elsewhere in the peritoneal cavity.

In the experiment with the streptococcus half the treated mice (5) had died by the end of the 4th day, and from these the streptococcus was recovered in pure culture from the heart blood and peritoneal fluid. The rest lived on and led us to entertain hopes of a 50 per cent. survival rate, but on the 12th, 15th and 16th days three more died. From these three only intestinal organisms could be isolated. The two which survived were lively, but their coats did not recover the normal sleekness for at least a month after the infection.

Table III.

Infecting organism.	Expt.		Single dose (mg.).	Number of doses.	Interval (hours).	Route.	Number of mice.	Survivors at end of day.										
								1.	1¼.	2.	3.	4.	5.	6.	7.	8.	9.	10.	20.
Staph. aureus	1	Control	..	..	..	..	10	4	2	..	1	0	..	..	..	..	..	..	..
		Treated	2	2	12	Intravenous	5	..	..	..	..	..	..	..	..	3	..	..	0
			10	1	..	Gastric	5	..	..	..	..	..	4	..	..	3	..	..	1
	2	Control	..	..	..	..	10	3	..	2	..	..	1	..	..	..	..	..	..
		Treated	1	2	12	Intravenous	5	..	..	4	..	3	1	..	..	..	..	..	..
			5	1	..	Gastric	5	4	..	2	1	..	..	0	..	..	..	..	..
	3	Control	..	..	..	..	10	3	0	..	..	..	..	..	..	..	..	..	..
		Treated	2	3	12	Intravenous	5	..	..	..	..	..	..	..	4	3	..	..	0
			2	4	12*	Intravenous	5	..	..	..	..	..	..	..	4	3	..	1	0
	4	Control	..	..	..	..	10	1	..	0	..	..	..	..	..	..	..	..	..
		Treated	10	1	..	Gastric	10	..	..	..	..	..	9	..	5	4	3	2	1
	5	Control	..	..	..	..	5	1	0	..	..	..	..	..	..	..	..	..	..
		Treated	10	8	12*	Gastric	5	..	4	3	..	..	..	1	0	..	..	..	..
Strept. pyogenes	..	Control	..	..	..	..	10	8	4	2	0	..	..	..	..	..	..	..	..
		Treated	2	10	12	Intravenous and subcutaneous	10	..	9	..	6	5	..	..	..	..	..	..	2

* 24 hours between last two doses.

A possible cause of the late deaths, after the infection had apparently been overcome, appeared when histological examination was made of two mice which had received parallel treatment with sodium helvolate without having suffered the streptococcal infection. The appearances are described in the section on toxicity.

Comment.

Helvolic acid is a substance which has been shown to have a powerful antibacterial action, mainly against Gram-positive organisms. It has no effect on the respiration of staphylococcal suspensions and its action is mainly bacteriostatic. It is relatively non-toxic to leucocytes and tissue cultures. A dose well below the lethal ensures a high antibacterial titre in the blood of mice and of a cat. It is absorbed from the gut, subcutaneous tissues and peritoneal cavity and excreted in the urine and bile.

In view of these properties when the mouse protection tests were undertaken it seemed probable that they would be successful. Though that hope was not realized it is fairly clear from the results that prolongation of life resulted. It will be noted that repeated large injections had a less beneficial effect than a single injection. In searching for an explanation for the failure of the therapeutic experiments, the influence on bacteriostasis of ground-up staphylococci and yeast, of p-amino-benzoic acid and the broth in which staphylococci had grown and of other biological fluids was investigated without revealing a sufficient cause. It had already been shown that the number of bacteria in an inoculum influenced the titre at which inhibition took place (though the exact cause for this still escapes detection), and as the first experiments were done with staphylococci, which require a numerically large dose of organisms, it was hoped that the failure to save life was thereby explained. Treatment of a streptococcal infection, where a much smaller dose of organisms was needed, was, however, hardly more successful. Finally, the histological investigation of controls injected with the drug at the same time as the infected animals gave clear-cut evidence that repeated injections caused severe liver damage, which explained why several large doses were less effective than one or two, and no doubt had much to do with the failure to save life.

It would thus appear that helvolic acid is a substance which hovers on the borderline of being an effective chemotherapeutic agent. It has some properties similar to those of the sulphonamides, e.g. the influence of the number of bacteria in the inoculum on the titre at which inhibition can be shown, but it differs from them in that its activity is not affected by peptone and p-amino-benzoic acid, and to only a limited extent by pus, serum and liver extract.

Chemically, it almost certainly belongs to a group of substances so far unexplored for their antibacterial action. If the substance can be synthesized it may prove of value as a local application to infected wounds, for it has lately been shown that more toxic substances, such as propamidine and proflavine, can be applied with success locally without general toxic symptoms (Thrower and Valentine, 1943 ; Mitchell and Buttle, 1942).

During the progress of this work Waksman *et al.* (1942), in a preliminary communication, reported the isolation of an antibiotic from culture filtrates of *A. fumigatus*, which they called Fumigacin. Since going to press a fuller account of the properties of this substance has appeared (Waksman *et al.*, 1943) and it is clear that it is not identical with helvolic acid, as there are several well-defined chemical differences, and in particular, fumigacin contains nitrogen.

SUMMARY.

1. A new antibiotic has been isolated in the crystalline state from culture filtrates of *Aspergillus fumigatus, mut. helvola* Yuill. It has been named helvolic acid, and has the elementary composition $C_{32}H_{44}O_8$.

2. Helvolic acid titrates as a monobasic acid, and a crystalline monomethylester has been prepared from it by the action of diazomethane.

3. Helvolic acid is soluble in most organic solvents, except petrol, but insoluble in water; its sodium salt is readily soluble in water.

4. The antibacterial activity of helvolic acid is not affected by heating to 100° for 15 minutes in 2N acid or at pH 7 or pH 10.

5. Helvolic acid is in general active against Gram-positive and almost inactive against Gram-negative organisms. The dilution at which it inhibits growth is influenced by the number of organisms in the inoculum. Its action is predominantly bacteriostatic.

6. The upper limit of intravenous dose of sodium helvolate tolerated by mice of 20 gm. is about 5 mg., while 20 mg. are tolerated by the gastric route (larger doses were not tried).

7. Leucocytes are unaffected by dilutions over 1 : 1600, and tissue cultures can be exposed to 1 : 2500 for 48 hours without suffering irreversible changes.

8. The substance is absorbed from the subcutaneous tissue and gastro-intestinal tract and is excreted in the urine and bile.

9. A high antibacterial titre can be produced in the blood, but repeated injections damage the liver.

10. In mouse-protection experiments prolongation of life can be shown.

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A NOTE ON THE CRYSTALLOGRAPHY OF HELVOLIC ACID AND THE METHYL ESTER OF HELVOLIC ACID.

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HELVOLIC acid, prepared as described in the preceding communication, was obtained in a microcrystalline state showing the same crystallographic form from a wide variety of solvents. By recrystallization from glacial acetic acid somewhat larger crystals were obtained. They form four and six-sided monoclinic needles elongated along the *b* axis. There was a marked cleavage plane parallel to the needle axis. The crystals gave rather poor X-ray photographs, from which the following measurements for the size of the unit cell were obtained :

$a = 14{\cdot}1 \pm 0{\cdot}05$, $b = 8{\cdot}32 \pm 0{\cdot}05$, $c = 31{\cdot}0 \pm 0{\cdot}2$A.

$\beta = 129°$.

Space Group A2, $n = 4$.

Density $1{\cdot}140 \pm 0{\cdot}01$.

Since the space group requires at least four molecules in the unit cell the molecular weight of helvolic acid is 554 ± 10 or some sub-multiple of this. This, combined with the analytical data, favours the formula $C_{32}H_{42}O_8$ (mol. wt. 556) for helvolic acid.

Helvolic Acid Methyl Ester.

Helvolic acid methyl ester, prepared as described in the preceding communication, was obtained in a microcrystalline state. Recrystallization from benzene and petroleum ether gave somewhat larger crystals. Crystals from both these preparations were faintly yellow four- and six-sided needles, elongated along the *b* axis. A cleavage plane was prominent along this axis. Although still small, the crystals gave very good sharp X-ray photographs. The following measurements were obtained for the size of the unit cell :

$a = 13{\cdot}74 \pm 0{\cdot}14$, $b = 8{\cdot}42 \pm 0{\cdot}05$, $c = 15{\cdot}48 \pm 0{\cdot}08$A.

$\beta = 116°$–$117°$.

Space Group $P2_1$, $n = 2$.

Density $1{\cdot}144 \pm 0{\cdot}006$.

The space group requires the presence of at least two molecules in the unit cell. From this, the measured cell dimensions and the density, the molecular weight may be calculated as 556 ± 14 (or as before, some sub-multiple of this). The calculated molecular weight for $C_{32}H_{43}O_8CH_3$ is 570, which does agree with this within the limits of experimental error.

Helvolic acid and helvolic acid methyl ester are strikingly similar crystallographically. They are practically indistinguishable by morphological and also optical measurements—in both β is parallel to the *b* axis, α to the *c* plane.

The cell dimensions are very similar with the exception that the difference in space group necessitates the doubling of *c* in helvolic acid compared with methyl helvolate. But in spite of this the actual molecular arrangements must be similar, since there is a striking likeness particularly in the intensities of the *hol* reflections of the two substances, which show an approach to pseudo-hexagonal symmetry.

SEROLOGICAL SPECIFICITY OF AN AUTO-ANTIBODY IN ATYPICAL PNEUMONIA.

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NOT long ago brief reports issuing independently from both sides of the Atlantic indicated the existence of a relation between atypical pneumonia and the cold agglutinins (Peterson *et al.*, 1943 ; Turner, 1943). More recently, a comprehensive investigation of the behaviour of the cold agglutinins in a variety of infectious diseases revealed the aspect of the subject which seemed to be the key to its clinical and immunological significance. This was the demonstration that the titre of cold agglutinins for the human erythrocyte rises in atypical pneumonia during the second week after the onset of respiratory symptoms, in the fashion of specific immune antibodies (Turner *et al.*, 1943). The establishment of this important temporal relation permitted the formulation of hypotheses to account for the phenomenon, and enabled us to show that abnormal amounts of cold agglutinin developed in more than 90 per cent. of a group of cases of atypical pneumonia seen in the British Isles during the winter 1942–43. Thus, even though the infectious agent in this disease remains unknown except in the negative sense that it is probably not bacterial (Smadel, 1943), a reasonable and simple serological principle for the delineation and classification of a current type of respiratory disease has been laid down. Furthermore, certain analogies to known specific immunological systems have been pointed out, and the idea advanced that in atypical pneumonia the cold agglutinin is likewise specifically related to the agents responsible for the disease (Turner *et al.*, 1943), either through the operation of heterogenetic elements, or by virtue of a complex antigenic stimulus, such as has been postulated for paroxysmal haemoglobinuria (Landsteiner, 1936). A precise and direct experimental investigation of the mechanisms at play must await the isolation of the etiological agent concerned. Meanwhile, however, some demonstration of the degree of serological specificity of the agglutinin in atypical pneumonia would appear to be highly desirable. For, in the first place, the agglutinins acting at low temperatures on the human erythrocyte are usually referred to as " non-specific " (Wiener, 1939). Moreover, the striking association between cold agglutinins and auto-agglutinins in paroxysmal haemoglobinuria and trypanosomiasis (Donath and Landsteiner, 1925 ; Yorke, 1910) serves to raise the question whether these antibodies are related to that of atypical pneumonia. From an entirely different view the problem of specificity contains considerable interest. We have drawn attention elsewhere (Turner, 1943) to an early contribution (Clough and Richter, 1918) which described a clinical and serological pattern strikingly like the one of atypical pneumonia in 1942. The thoroughness of the studies makes possible a direct comparison of the range of biological activity of the agglutinins found then and now. Any similarities would further support the idea that the patient seen 25 years ago represented a sporadic case of what is now

epidemic atypical pneumonia. We have therefore examined the cold agglutinin of atypical pneumonia for serological specificity and the results are herewith presented.

Serological specificity has been defined as the " disproportional action of a number of similar agents on a variety of related substrata " (Landsteiner, 1936, p. 6). Among the countless investigations of species and individual specificity of antigens and antibodies, erythrocytes have been so extensively employed as substrate that the reactions involving haemagglutinins and haemolysins lend themselves as a rule to ready interpretation. Blood cells of species and invididuals can be distinguished by the differential action of normal or immune sera. On the other hand, complex mixtures of antibody may be partitioned by absorption with various appropriate cell types, and sera possessing even a wide range of action upon a variety of antigens may thus be shown to be equipped with more or less specificity. It is possible to demonstrate such dissimilarities by the employment of simple techniques, notably titration and absorption, and these standard methods were used in the present study. Sera of patients with atypical pneumonia are compared with control sera in their content of cold agglutinins for the erythrocytes of the human genotypes and a number of animals.

Materials and Methods.

Venous blood was withdrawn from the patients and, except as may be otherwise indicated, the serum was allowed to separate at room temperature. Within 24 hours thereafter the agglutination tests were set up with fresh erythrocytes washed three times in saline solution. Serial dilutions of 0/3 ml. serum in equivalent amounts of ·85 per cent. solution of sodium chloride were made in Kahn test-tubes, the range of titre being from 1 : 2 to 1 : 1024. To each series of tubes was added 0/1 ml. of a 3 per cent. suspension of the type of cells designated. The racks were set in a refrigerator maintaining a temperature of about 5° C., and examinations for agglutination made the following morning after an interval of about 18 hours. A first reading was taken immediately upon removal of the tubes from the cold, a second after 2 hours at room temperature, and a third after the tests stood for 2 hours in an incubator at 37° C. It was thus possible to obtain an approximation of the thermal amplitude of activity of agglutinin. The end-point of highest titre was defined with the aid of a hand lens after the deposit of cells had been resuspended in the fluid by rapid back-and-forth rotation of the tube while it was being held vertically between the palms. This is essentially the method commonly employed to read specific immune agglutinations, such as that of sheep cells in the well-known test for infectious mononucleosis.

The cases of atypical pneumonia represented clinically characteristic examples of this disease, and the diagnosis was confirmed by X-ray examination of the lungs.

Cold Agglutinins as Auto-antibodies.

Serum from atypical pneumonia is found to have an approximately equivalent effect upon the cells of the individual (autologous) from whom it is derived and upon homologous group O cells. In both cases the action is most powerful in the cold, falls off sharply at room temperature, and disappears at 37° C. Table I indicates the comparative titres found in a number of sera. There is often a slightly higher titre for homologous cells, but in the view of the errors of the method this difference may be more apparent than real. On the other hand, it is also possible that it is due to an additional minor component of antibody. In view of these findings it seems appropriate to look upon the cold agglutinin in atypical pneumonia as an auto-antibody. It will be shown later that the agglutinin is absorbed by autologous cells.

The low titre obtained with a normal serum calls attention to the so-called non-

TABLE I.—*Comparative Action of Cold Agglutinin on Autologous and Homologous Erythrocytes.*

	Serum from patients with atypical pneumonia.							Normal serum.
	V.	R.	F.	M.	G.	P.	W.	D.
Autologous cells . . .	256	512	512	256	64	128	256	4
Homologous cells—Group O .	512	256	1024	128	128	512	512	4

Numbers indicate titre at 0°–5° C.

specific cold or auto-agglutinin which is found in small amounts in the blood of many healthy individuals. With the method employed we have found the titre of the normal agglutinin to be below 1 : 16 except in very rare instances.

Cold Agglutinins as Hetero-Agglutinins.

In order to gain a first approximation to the specificity of the agglutinin for the human erythrocyte, the range of activity of serum upon cells of various animals was studied. Sera from two cases of atypical pneumonia as well as from several controls

TABLE II.—*Cold Agglutinins of Atypical Pneumonia as Distinct From Hetero-agglutinins.*

	Cells of—					
Serum of—	Human group O.	Rabbit.	Mouse.	Guinea-pig.	Horse.	Sheep.
W. (a.p.)	1024	1024	1024	512	64	64
P. (a.p.)	512	1024	128	256	64	32
T. (pn.pn) . . .	8	1024	128	128	64	16
D. (head cold) . . .	4	1024	128	256	256	32
A. (normal control) . .	0	512	128	64	128	16
B. (normal control) . .	0	1024	64	64	64	16
C. (normal control) . .	0	512	64	128	64	32
E. (normal control) . .	2	512	128	16	512	16
F. (normal control) . .	0	512	128	1024	16	128
G. (normal control) . .	0	1024	256	128	128	128

Numbers indicate titre at 0°–5° C. (a.p.) = atypical pneumonia ; (pn. pn.) = pneumococcus pneumonia.

were tested in this manner, and the results may be seen in Table II. It is apparent that while the activity of the serum from atypical pneumonia extends to the cells of all species examined, equally powerful hetero-agglutinins may be found in control sera which contain little or no cold agglutinins for human O cells. Evidently there is in most human sera a complex mixture of haemagglutinins which act at low temperatures, and are present in such high degree that titration of the serum will not suffice for the delineation of specificity. The thermal amplitude of the agglutinins for the animal cells varied to some extent, but there was, invariably, considerably more activity at low temperatures. We therefore turned to the method of selective absorption, in order to separate the cold agglutinin for human cells.

Isolation of Auto-agglutinin.

Among techniques employed for the purpose of obtaining antibody in as pure a state as possible, absorption by appropriate substrata and subsequent dissociation of antigen-antibody complex has been extensively used. In systems involving cells, such as the one under consideration here, reversibility of the union of antigen and

antibody is often accomplished by the application of heat (Zinsser *et al.*, 1939, Chap. V). In particular, cold agglutination of erythrocytes may be reversed by warming to 37° C.—a procedure which appears to injure neither antibody nor substrate, since the agglutination recurs if the system is transferred again to a temperature of 0°–5° C. These considerations have been successfully applied to the refinement of the cold agglutinin in atypical pneumonia.

Auto-agglutinin was isolated by a series of manipulations carried out at low temperatures. Whole defibrinated or oxalated venous blood was allowed to stand in an ice-bath for several hours, a procedure designed to permit the absorption of auto-antibody by the cells. After removal of the supernatant, the erythrocytes were washed three times with cold saline. An amount of saline was then added sufficient to restore original volume and the mixture was placed at 37° for 2 hours in order to dissociate the red cell agglutination. The supernatant recovered after final centrifugation is referred to as "saline solution of auto-agglutinin."

Specificity of Auto-Agglutinin.

Serial dilutions of the saline solution of auto-agglutinin were tested in the same manner as serum. The results of several experiments are exhibited in Table III. The titres for human cells are somewhat lower than those found with the corresponding serum, doubtless in large part because the antibody is incompletely recovered. It may be seen, however, that considerable amounts of auto-agglutinin can be isolated by this method. Furthermore, there is a degree of serological specificity inasmuch as the action upon human cells and rabbit cells is about equal, whereas less affinity is shown for the erythrocytes of other animals. No agglutination whatever is exhibited by the cells of horse or ox. It should be noted that the titres for both human and rabbit cells fell to 1 : 4 at room temperature, and no agglutination was seen at 37° C. The close relation of rabbit to human cell in this system was further shown by absorption of the saline solution of auto-agglutinin with rabbit erythrocytes. A single absorption at 0° C. with an equal volume of cells reduced the titres for both human and rabbit cells to 1 : 4.

TABLE III.—*Auto-agglutinin as Iso- and Hetero-agglutinin.*

Saline solution of the auto-agglutinin of—	Cells of—								
	Human		Rabbit.	Guinea-pig.	Pig.	Sheep.	Horse.	Mouse.	Ox.
	O.	AB.							
W. (a.p.) (1024) . .	256	128	256	16	16	8	0	0	0
S. (a.p.) (1024) . .	256	256	256	128	32	0	0	32	0
B. (a.p.) (128) . .	128	128	256	32	16	2	0	2	0
L. (a.p.) (128) . .	64	64	64	8	8	0	0	0	0
H. (a.p.) (64) . .	32	32	32	8	8	0	0	0	0
C. (a.p.) (32) . .	16	16	16	8	4	0	0	0	0
A. (a.p.) (256) . .	32	16	64	4	0	0	0	0	0
J. (normal) (4) . .	2	4	2	0	4	0	2	0	0

Figures in brackets indicate titres of fresh, unabsorbed serum for human O cells.

The effect of the removal of auto-agglutinin from serum upon the equipment of hetero-agglutinins may be seen in Table IV. Together with the expected pronounced fall in titre for human cells, there would appear to be some regular decrease in titre

TABLE IV.—*Iso- and Hetero-agglutinins of Atypical Pneumonia before and after the Removal of Auto-agglutinin by Absorption.*

Serum.	Cells of—					
	Human type O.	Rabbit.	Mouse.	Guniea-pig.	Horse.	Sheep.
P Unabsorbed .	512	1024	128	256	64	32
Absorbed with autologous cells .	8	1024	64	32	128	32
S Unabsorbed .	1024	512	64	1024	32	256
Absorbed with autologous cells .	16	256	64	128	16	64
B Unabsorbed .	128	512	32	256	16	32
Absorbed with autologous cells .	2	128	16	64	16	16
L Unabsorbed .	128	256	256	128	16	32
Absorbed with autologous cells .	4	256	256	64	8	16
H Unabsorbed .	64	512	64	128	32	64
Absorbed with autologous cells .	8	128	32	32	32	64
C Unabsorbed .	32	256	128	128	32	32
Absorbed with autologous cells .	2	128	32	64	32	32

for guinea-pig cells, while the affinity for the erythrocytes of the other animals represented is not materially affected. Inasmuch as the solution of auto-agglutinin has been seen to agglutinate guinea-pig cells (Table III), it is not surprising that the absorbed serum shows a corresponding loss in activity. One might anticipate similar findings in the case of rabbit cells, for upon them the auto-agglutinin has an even more powerful effect. But the situation appears to differ in that the serum after absorption shows no significant change in titre for rabbit cells. Apparent inconsistencies of this kind are not unknown, and have not been satisfactorily explained except in so far as they reflect errors of method and the operation of complex characters possessed of only a relative specificity that varies in degree (Landsteiner, 1936, Chap. IV). It is our purpose here only to indicate that the cold agglutinin in atypical pneumonia may be absorbed by appropriate substrate, and that it then behaves with relative, not absolute, specificity.

No differences in susceptibility to agglutination were disclosed among the four major human blood groups. The genetic elements symbolized by the allelomorphs A, B and O thus appear to play no role in the reaction. Facilities have not been available to permit a precise account of the behaviour of the known minor agglutinogens in this respect, but the regularity with which the cold agglutinin has appeared in the many cases of atypical pneumonia seen by us makes it unlikely that the phenomenon is related to these substances.

DISCUSSION.

The cold agglutinin in atypical pneumonia has been seen to be selective enough to fulfil the criteria for specificity defined by Landsteiner (1936), who has been quoted earlier. For, when isolated as an auto-agglutinin from the mixture of antibodies in the serum, its action is most pronounced upon the erythrocytes of man and rabbit among eight species tested. It is the rule, rather than the exception, to find relative and not absolute specificity in such systems, inasmuch as chemically similar antigens may produce effects indistinguishable by the techniques employed. The fact that the two types of erythrocyte here derive from widely separated members of the animal kingdom suggests at once the operation of heterogenetic elements analogous to the Forssman antigens. A heterogenetic relation between red blood cell and the as yet

unknown infectious agent responsible for atypical pneumonia could be proposed as an explanation of the peculiar serological abnormality in this disease.

Comparison of the findings presented herewith and those obtained by Clough and Richter in a case of broncho-pneumonia seen in 1918 indicates striking serological similarity. Their patient developed an auto-agglutinin which had a powerful effect on rabbit as well as human erythrocytes. Moreover, absorption with either cell type removed agglutinins for both. The specificity is thus remarkably like that exhibited by the cold agglutinin of current atypical pneumonia, and can be taken as some evidence for the belief that atypical pneumonia may have existed in sporadic form for many years before it became epidemic.

It should be added that there remains to be investigated what is perhaps the most significant aspect of the problem of specificity, to wit, the extent to which known viruses of the respiratory tract, e.g. psittacosis, influence the titre of cold agglutinin. Infection by influenza virus " B " would appear to be unrelated to the phenomenon (Turner *et al.*, 1943), and as more such agents are studied the specificity for non-bacterial organisms can be formulated to some extent even in the absence of accurate knowledge of the etiological agent in atypical pneumonia.

SUMMARY.

1. The cold agglutinin developing during the course of atypical pneumonia has been studied for serological specificity by the methods of selective absorption and titration of activity upon the erythrocytes of several species.

2. The agglutinin is shown to have the properties of auto-antibody, and reacts equally with cells representing all four major human blood groups.

3. When isolated by absorption, the auto-agglutinin exhibits a limited action upon the erythrocytes of lower animals, having appreciable effect upon rabbit cells only.

4. It is suggested that this specificity may be explained by the existence of a heterogenetic antigen shared by man and rabbit.

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SOME INVESTIGATIONS UPON THE NATURE OF THE RESISTANCE OF AN INBRED LINE OF FOWLS TO THE DEVELOPMENT OF THE ROUS No. 1 SARCOMA.

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UNDER a scheme sponsored by the Scientific Advisory Committee of the British Empire Cancer Campaign, the fowls of the pedigreed Brown Leghorn flock of the Institute of Animal Genetics were made available to workers using avian tumours. The susceptibility of the various individuals was noted and the results analysed. It became clear that parentage was an important factor in determining the degree of susceptibility of 6-week-old chicks to inoculation with the Rous No. 1 sarcoma agent, and it was found possible by selection to produce a line of birds, known as the Non-Susceptible or N-S line, which was very resistant to inoculation with the Rous No. 1 agent. The genetical aspects of this work will be dealt with in separate publications by Dr. Greenwood. The present account deals with some preliminary investigations into the nature of this resistance.

It should be emphasized that the N-S line was established and tested before the breeding stock had ever been in contact with birds bearing experimental tumours. Furthermore, spontaneous neoplastic diseases are extremely rare in the Institute flock. Acquired immunity resulting from infection with experimental material or spontaneously developed tumours (either of the animal or its parents) could thus be ruled out as complicating factors.

Response of N-S Birds to Inoculation with Tumour Agent.

All N-S birds are tested for susceptibility to Rous No. 1 agent at the age of 6 weeks (see Carr, 1942*a*, for details). In Table I is shown as an example of the difference between the response of this line and " controls " (i.e. birds taken at random from other Institute lines) data obtained over a period of 1941. The difference between the two is slightly reduced, because it was found necessary to kill about 11 per cent. of the control birds before maximal tumour development in order to provide material for experiments. Seasonal variation in response is shown neither by the N-S nor by the " control " birds (Carr, 1942*c*). The sexes contribute approximately equally to each group.

TABLE I.—*Showing Degree of Response of 6-week-old N-S and Control Birds to Inoculation with Rous No. 1 Agent.*

	Number tested.	Tumour response as percentage ++++.	+++.	++.	+.	Regression.	Negative.
N-S line .	128	0	0	1	28	63	8
Controls .	118	18	25	33	16	7	0

As a general rule the growth of the tumours in both the N-S and control fowls is similar for about the first 14 days. The N-S tumours then appear to lag behind the controls ; many become distinctly harder than the controls and remain about + in size, while the rest suddenly soften and completely or almost completely disappear by about the 22nd day. In the case of these regressions, a small tumour sometimes persists at the surface of the muscle or in the skin ; these are not classified as regressions. In the majority of birds regression appears complete, as far as can be judged macroscopically at post-mortem examination. Those tumours which persist in the N-S birds show a very slow and steady growth or regression. The results are independent of the amount of agent given, over the ranges used (10–1,000 minimal infective doses).

Effect of Age on Resistance.

In a previous paper (Carr, 1942*a*) a fall in the resistance of older N-S birds was described, which in several instances led to a redevelopment of Rous tumours without further inoculation. A series of susceptibility tests were carried out upon a number of day-old chicks, employing the same technique as for the 6-week-old fowls. As Table II shows, the tumour grew more vigorously in both N-S and control day-olds than in the older birds, but the N-S birds were still obviously less susceptible than the controls.

TABLE II.—*Showing Response of N-S and Control Birds Inoculated with Rous No.* 1 *Agent as Day-olds.*

	Tumour response.					
	++++.	+++.	++.	+.	Regression.	Negative.
N-S line .	0	7	8	15	11	3
Controls .	6	10	1	0	0	0

Metastases were frequently found in both groups, whereas they have not been noted in N-S fowls inoculated at the age of 6 weeks. It was observed that tumours sometimes appeared in the day-old birds at inoculation sites which parallel tests on 6-week-old animals showed to be quite devoid of active agent. As a control, some day-olds were injected with Rous agent into the breast and saline into the legs. A few tumours appeared at the site of the saline injections, indicating that the anomalous tumours were secondaries forming at points of injury. It should be noted that, as a consequence of this, comparative tests of two or more agent preparations in one animal are unreliable if day-old chicks are used.

Relation of Antibodies to Resistance.

At the beginning of this work the possibility that serum antibodies or other serum neutralizing factors might be the cause of this resistance was envisaged. Accordingly, experiments were early undertaken (while working at the Lister Institute, London) to investigate this point, before any of the tested fowls were returned to Edinburgh for breeding purposes, thus avoiding any complications due to immunity resulting from transmission of tumour agent to the experimental birds. The details of one such experiment will be described, and the others, which gave the same result, summarized.

Experiment 1.

Blood was taken from the wing vein of N-S fowls L.355 and L.367, and from control fowls L.397 and L.411, immediately on receipt from Edinburgh. All were 6 weeks old. The sera obtained were used in the neutralization test.

A cell-free Rous No. 1 agent preparation was inoculated into these fowls in the usual manner employed for testing susceptibility, and the same preparation was used for testing the neutralizing power of the sera.

Susceptibility results :

L. 397. Tumour size ++++, 30 days after inoculation with agent.
L. 411. ,, ,, ++++, ,, ,, ,,
L. 355. ,, ,, +, ,, ,, ,,
L. 367. No tumours resulted.

The suspension of agent used produced tumours in all of three susceptible birds when diluted 1/10, and in 1 out of 3 when diluted 1/100 ; 0·5 c.c. was inoculated.

Serum test : Mixtures of 2 vols. of undiluted Rous agent suspension + 1 vol. serum were incubated for 1 hour, and 0·5 c.c. inoculated into 6-week-old " controls " as shown in Table III.

TABLE III.—*Showing Absence of Neutralizing Antibodies in N-S Sera.*

The size of the tumours is indicated by the number of + signs.

Inoculum.			Site.	Fowl No. 942.	943.	949.
Agent suspension + serum		L.397	Right breast	+++	*	+
,, ,,	,,	L.355	Left ,,	+++	*	+
,, ,,	,,	L.397	Right leg	++++	*	+
,, ,,	,,	L.367	Left ,,	++++	*	+
				945.	946.	947.
,, ,,	,,	L.411	Right breast	+++	+++	+
,, ,,	,,	L.355	Left ,,	+++	+++	+
,, ,,	,,	L.411	Right leg	++++	+++	+
,, ,,	,,	L.367	Left ,,	++++	+++	+

* Indicates tumours regressed.

It is obvious that there were no detectable neutralizing antibodies in the N-S sera. Similar experiments on other fowls, carried out in essentially the same way as Expt. 1, always produced the same result. These are summarized below.

Expt. 2. Fowl L.298, susceptibility +, compared with L.497, susceptibility ++++.

Expt. 3. Fowls L.1843 and L.1938, susceptibility 0, each compared with L.1764, L.1916 and L.1933, susceptibility of each ++++.

Expt. 4. Fowls L.2394 and L.2398, susceptibility +, each compared with L.2143 and L.227, susceptibility of each ++++.

Expt. 5. Fowl L.2356, susceptibility +, compared with L.2397, susceptibility ++++.

All these experiments failed to give any indication that N-S sera contained any neutralizing substance not present in the sera of susceptible birds. A further test was carried out after the introduction of some tested fowls into the N-S breeding stock, the serum on this occasion being taken from the chicks 7 days after the testing dose of Rous agent was inoculated. Once again the N-S sera failed to inhibit the activity

of a Rous agent suspension. It thus seems clear that preformed antibodies cannot be the cause of the resistance of these birds.

Although antibodies to the Rous No. 1 agent are often present in " normal " fowls, they are usually found only in older animals (Andrewes, 1931 ; Ledingham and Gye, 1935 ; Amies, 1937 ; Duran-Reynals, 1940). Amies found no indication of their presence in an extensive series of young chicks. Such antibodies would rather be expected to result in failure to produce a tumour after inoculation of agent, whereas Table I indicates that the N-S line characteristically produces regressing tumours. Furthermore, the breeding experiments have indicated that the sire is an important factor in determining the degree of resistance of the chicks. This would not be expected if the resistance were due to a neutralizing substance transmitted from the dam *via* the egg. These facts provide additional evidence that antibodies are not the cause of the resistance exhibited by the N-S line.

Transplantation of Rous Cells into N-S Birds.

If the failure of the Rous tumour to develop in the N-S birds were due to some factor in the cells which was inimical to their cancerous development, then transplants of tumour cells from a progressive tumour should result in proliferation of the graft. Implants of cells from routine Rous No. 1 tumours into 14 N-S birds whose agent-induced tumours had regressed showed undoubted growth in only one case. It might be objected that these grafts were eliminated by iso-antibody action. Opportunity was therefore taken of the finding that progressive tumours may sometimes appear in N-S fowls after treatment with acenaphthene or methylcholanthrene to transplant progressive N-S tumours, but without any greater success (Carr, 1942*b*). The cause of the resistance is thus not to be found in the induced tumour cells. The reverse experiment of grafting N-S tumours into control fowls was not considered practicable, owing to the possibility of infecting the host cells with agent liberated from damaged tumour cells.

Growth of Other Tumours in N-S Fowls.

By the courtesy of Dr. Peacock, a specimen of the chemically-induced GRCH/15 sarcoma was obtained, and has been maintained at this Institute. All control fowls inoculated with cells of this tumour have grown progressive tumours. Grafts into 25 N-S birds whose agent-induced tumours had regressed resulted in only 14 birds yielding progressive tumours, and grafts into 2 untested N-S birds grew slightly and then regressed. The tumours which did grow all resembled typical GRCH/15 sarcomas in rate of growth, macroscopic and microscopic appearance. The N-S line thus shows a distinct resistance to the growth of this non-filterable sarcoma as well. Unfortunately the fowls thus tested were offspring from 11 dams, so that the data is insufficient to show whether these differences in response were due to inherited factors. But it was noted that of the two dams who had 4 offspring tested, L.352's offspring all showed tumour growth, while those of L.1848 failed to do so. L.352 was related to the N-S line, but had not been tested herself. Her offspring were all as resistant to Rous agent as N-S fowls.

In order to avoid the risks of cross-infection no other avian tumours are being maintained, but a consideration of susceptibility results obtained in earlier work indicated that a high proportion of fowls resistant to the Fujinami sarcoma and the Des Ligneris sarcoma (which is very similar to the Fujinami (Amies, Carr and Purdy, 1939)) were related to the N-S line.

DISCUSSION.

In spite of a large amount of experimental work, little is really known about the subject of resistance to neoplastic growth. The resistance often exhibited to transplanted tumours is now considered to be due chiefly to iso-antibody action, and hence can offer no possibility of leading to any measures of therapeutic value for the treatment of spontaneous neoplasms. The study of the susceptibility of mice to spontaneous mammary neoplasms has shown that this is an exceedingly complex phenomenon involving hormones, milk-transmitted factors, maternal influences and chromosomal factors, whose disentanglement is only beginning. Investigations upon resistance to the action of chemical carcinogens have scarcely started. The N-S line of fowls thus represents a very valuable additional line of attack upon the problem of resistance and susceptibility to cancer.

The present work is only a preliminary orientating investigation into the nature of this resistance, about which little can yet be said. But the fact that the resistance is so obviously directed against *developing* tumours makes it of extreme interest from the point of view of possible therapeutic applications, which is enhanced by the suggestion that it is not confined to a single type of tumour.

As the N-S line was established solely on the basis of experimentally determined resistance to the Rous No. 1 agent alone, it is difficult to believe that an independent resistance to Fujinami and GRCH/15 tumours was unwittingly fixed simultaneously. It seems much more probable that a general resistance to tumour growth is involved. A fuller discussion of the data bearing upon this point will be given in the descriptions of the breeding experiments. Such a general inherited resistance to neoplasms should be of great interest to the poultry industry, in which losses due to such diseases are notoriously high.

It is interesting that the birds show their greatest resistance to Rous tumour agent at the age of about 6–10 weeks, at which time the animal itself is growing most rapidly. The term " resistance " is, of course, a compound of many factors. For instance, a tumour reaching a weight of 10 g. is a burden to a 3-week-old chick, but of little consequence to an adult ; differing rates of vital processes between young and old fowls may result in varying rates of growth of established tumours, and varying reactions to the toxic effects of necrotic portions of tumour. All these will influence the degree of resistance of the bird, and should be separately evaluated in a discussion of the variation of resistance with age. A simple variation need not be expected, and the data presented is not suitable for an elaborate consideration of this aspect of the problem.

The type of variation noted is, however, not consistent with the suggestion that the resistance of the N-S chicks is due to any protective substance transmitted from the dam *via* the egg, in which case the youngest chicks would be expected to have the greatest resistance. It was fortunate that the separation of the breeding and experimental work in the early days permitted a test at this point without any of the possible complications arising. All the evidence available suggests that such protective substances play no part in the resistance shown by the N-S birds. That resistance can exist independently of protective serum antibodies has been demonstrated for isolated cases by other workers (Andrewes, 1931 ; Duran-Reynals, 1940 ; Troisier, 1934). This, of course, does not mean that such antibodies may not in any circumstances contribute to host resistance. But the resistance of the young N-S fowls must be primarily due to other, as yet unknown, factors.

SUMMARY.

Inoculation of Rous No. 1 agent into 6-week-old fowls of the N-S line typically

produces small tumours, most of which regress between the 14th and 22nd day after inoculation. This resistance is present in day-old chicks, but to a lesser extent, and is decreased also in older birds. It is not due to serum antibodies. The resistance is exhibited also to implanted Rous cells, GRCH/15 cells, and possibly to other filterable tumours.

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